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(54) Polypeptides having thyrotropin receptor activity, nucleic acid sequences coding for such receptors and polypeptides, and applications of these polypeptides

Polypeptide mit Thyrotropinrezeptor-Aktivität, kodierende Nukleinsäuresequenzen für solche Rezeptoren und Verwendung dieser Polypeptide

Polypeptides possédant l'activité réceptrice de la thyrotropine, séquences d'acides nucléiques codant pour de tels récepteurs et polypeptides, et applications de ces polypeptides

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Description

The invention relates to a process for the preparation of recombinant polypeptides having thyrotropin-receptor activity, to nucleic acids coding for such polypeptides, and to the use of the polypeptides as obtained by the process in assay methods.

The literature references indicated by numbers in parentheses in this specification are listed in the form of a bibliography at the end of the description.

Pituitary glycoproteins (Luteinizing hormone, LH; follicle stimulating hormone, FHS ; and thyroid stimulating hormone or thyrotropin, TSH) form a family of closely related hormones.

The pituitary hormone thyrotropin (TSH) is the main physiological agent regulating the thyroid gland. It stimulates the function and the proliferation of thyrocytes and induces the expression of differentiation (1). Most of its effects are mediated by cyclic AMP (cAMP) (1). As the other pituitary and placental glycoprotein hormones (FSH, LH, CG), TSH is a heterodimer. All these hormones share an identical alpha subunit ; the beta subunit, despite sequence similarity, is specific for each (2). The activated TSH, FSH and LH-CG receptors stimulate adenylyl cyclase in their target cells via mechanisms mediated by the G protein Gs (3). In man, the TSH receptor may be the target of autoimmune reactions leading to hyper- or hypo-stimulation of the thyroid gland by autoantibodies in Grave's disease and in idiopathic myxoedema, respectively (4).

A prerequisite to studies of such diseases and to the elucidation of receptor structure and function is the availability of receptor preparations, particularly human, at a reasonable cost and in relative abundance.

To date, particulate membrane preparations and detergent-solubilised thyroid membranes, often of porcine or bovine origin (4) and (31) or (32) have been used in such studies. Human receptor preparations are not only costly but are also difficult to reproduce identically. Furthermore, the known preparations cannot be considered to be "purified" receptors; they are enriched with respect to their receptor content but do not allow purification of the receptor to a degree which would enable a partial sequence analysis, and hence its cloning.

Cloning and expression of the related LH-CG receptor has recently been achieved. A cDNA for the rat LH-CG receptor was isolated with use of a DNA probe generated in a polymerase chain reaction with oligonucleotide primers based on peptide sequences of purified receptor protein (15). Variants of the porcine LH-CG receptor were cloned by screening a λgt11 library with cDNA probes isolated with monoclonal antibodies (16).

Attempts have been made to clone the TSH receptor (6) using a method which exploits the sequence similarity displayed by all known G-protein coupled receptors. A pair of oligonucleotide primers corresponding to transmembrane segments III and VI were used on cDNA from thyroid tissue under conditions allowing amplification of the primed sequences by the polymerase chain reaction. The method did not allow cloning of the TSH receptor but led instead to the cloning of four new members of the G-protein coupled receptor family.

Various attempts to prepare monoclonal antibodies which recognize the TSH receptor are reported in the scientific literature as well. (33) describes monoclonal antibodies which are obtained from the fusion of human lymphocytes with myeloma cells and which are proposed as recognizing the TSH receptor because they interfere with TSH binding to human thyroid membranes and weakly stimulate cAMP production in FRTL-5 cells. However, all attempts to use said antibodies for the purification or cloning of the TSH receptor have failed.

(34) describes the preparation of TSH anti-idiotypic antibodies as monoclonals which are reported to recognize the TSH receptor. However, the nature of the molecules recognized by the antibodies described in (34) cannot be clearly derived from the Western blot experiments, and the authors in (34) could not identify the nature of the antigen recognized by their antibodies.

(35) describes the use of "antiparatypical antibodies" for detecting and treating autoimmune diseases, i.e. the use of antibodies which are raised against specific paratopes of other antibodies, i.e. against immunoglobulins. The use of antiparatypical antibodies is proposed in view of the difficulties in obtaining defined pure antigens as e.g. the TSH receptor.

(36) - a not prepublished international patent application, which claims a first priority of 8.9.1989 - reports the isolation of a cDNA clone which appears to represent a full length clone of the human TSH receptor. A deposit of said clone tr.12.6-1 was made on September 6, 1989. It was given the ATCC accession number 40651.

The present inventors have succeeded in cloning the TSH receptor and variants thereof, firstly by applying the technique described in (6) but with different sets of primers, and with human genomic DNA as the template, and secondly by use of a selected sequence amplified by this technique as a probe.

Certain aspects of the invention are illustrated in the figures 1 to 12. Figures illustrating amino-acid sequences use the one-letter abbreviation system.

Figure 1 is a sequence comparison of clone HGMP09 with a panel of G-protein coupled receptors ((6) and ref. therein). Only the sequence around transmembrane segment III of each receptor is shown in the one letter code. Residues conserved in HGMP09 and in more than 50 % of the other receptors are indicated by an asterisk. The "DRY" and "Asp113" residues (9) are indicated by ^.

Figure 2a shows the primary structure of the dog TSH receptor, as deduced from the nucleic acid sequence of dTSHr. The sequence was aligned (17) with full-length rat and pig LH-CG sequences (15, 16) and with HGMP09 partial sequence. Numbering is given from the first residue predicted in the mature polypeptide by von Heijne algorithm (11). Identical residues and conservative replacements in TSHr and LH-CGr are indicated by * and ., respectively. Sites for N glycosylation are underlined. Putative transmembrane segments are overlined. Lambda phages containing dTSHr inserts were subcloned in M13 and sequenced on both strands (Applied Biosystems model 370A) using a combination of forced cloning and exonuclease III deletions (21).

Figure 2b is a dendogram constructed from the sequences of G-protein coupled receptors. The CLUSTAL algorithm (17) was used to construct a dendogram from the sequences of 22 receptors ((6) and references therein) including rat and pig LH-CG receptors (16, 17), HGMP09 and the TSH receptor. For each receptor, a segment corresponding to the known sequence of HGMP09 (131 residues, extending from transmembrane segments II to V) was used for comparison by the program.

Figure 3a shows TSH induced morphological changes in Y1 cells microinjected with TSH receptor mRNA. Y1 cells were microinjected with recombinant TSH receptor mRNA (0.1 pl at 0.25 ug/ul) (right) or water (left) as described (13) and incubated in control medium (upper panel) or with TSH (0.1nM) (lower panel). RO 201724 and isobutylmethylxanthine (10^{-6} M each) were present in all incubations.

Figure 3b shows TSH induced cAMP accumulation in Xenopus oocytes microinjected with TSH receptor mRNA. Xenopus oocytes were handled as described (22) and injected with water (open symbols) or recombinant TSH receptor mRNA (13) (50 nl at 0.1 ug/ul) (filled symbols). After 3 days in control medium, batches of 35 oocytes were incubated for 90 min. in medium supplemented with various concentrations of TSH (circles), LH (squares) or FSH (triangles). cAMP was determined as described (14). RO 201724 and isobutylmethylxanthine (10^{-6} M each) were present in all incubations. Incubation of control oocytes in forskolin at 10^{-4} M resulted in doubling of the cAMP concentration (not shown).

Figure 4 illustrates the displacement of ^{125}I TSH receptors expressed in cos7 cells. Cos7 cells were transfected with TSH receptor cDNA subcloned in pSVL (23). After 72 hours, cells were harvested and a membrane fraction was prepared (24). Membranes were similarly prepared from wild type cos7 cells and from dog thyrocytes in primary culture (20). Binding of ^{125}I TSH (TRAK Henning) was performed at 0°C for 120 min. in the presence of various concentrations of competitors (TSH-Armour, FSH and LH, UCB bioproducts). Bound radioactivity was separated by centrifugation and counted. Results are expressed as percent ^{125}I TSH bound by transfected cells in the absence of competitor (3,000 cpm) over nonspecific binding (radioactivity bound in the presence of 100nM cold TSH, 800 cpm). Open and filled circles represent displacement by cold TSH from cos7 and thyrocyte membranes respectively. Open and filled squares represent displacement from cos7 by LH and FSH, respectively. Diamonds represent control cos7 cells in presence of various amounts of cold TSH.

Figure 5 shows the cDNA sequence coding for the dog TSH receptor, which was expressed in oocytes and culture cells.

Figure 6 is schematic representation of the dog thyrotropin receptor, showing the 7 putative transmembrane segments and the large NH2 terminal extracellular domain (to the exclusion of the signal peptide). The amino-acids deleted in the variant form are indicated in black. The five putative glycosylation sites are shown.

Figure 7 shows the sequence alignment of the repeats constituting the extracellular domain of the thyrotropin receptor amino-acid sequence. The signal peptide, as determined by Von Heijne algorithm is represented in italic. The repeat missing in the molecular variant of the receptor is indicated by the leftward arrow.

Figure 8 shows the primary structure of the human TSH receptor as deduced from its cDNA sequence. The amino-acid sequence corresponds to the 2292 nucleotide open reading frame determined from the sequencing of two overlapping inserts in lamda gtII clones (see examples). It is aligned for comparison with the dog TSH receptor sequence (only non conserved amino-acids are indicated). Numbering starts from the first residue of the mature polypeptide as determined by von Heijne algorithm [11]. Potential N-glycosylation sites are underlined and putative transmembrane segments are overlined.

Figure 9 shows the displacement by nonradioactive TSH of [^{125}I]TSH from human TSH receptors expressed in cos-7 cells. Results are expressed as percentage of the [^{125}I]-labelled TSH bound by transfected cells in the absence of competitor (1400 cpm) after correcting for nonspecific binding (radioactivity bound in the presence of 100 nM unlabelled TSH, 240 cpm).

Figure 10 represents the displacement by immunoglobulins of [^{125}I]TSH from human TSH receptor expressed in cos-7 cells. Results are expressed as described in the legend to fig. 9. Immunoglobulins were prepared (see examples) from a normal individual (N), from patients with idiopathic myxoedema (IM1, IM2) or Graves' disease (GD1, GD2). The concentration of immunoglobulins in the assay is indicated. The ability of IM1 and IM2 (1.5 mg/ml) to inhibit TSH-stimulated cAMP production in a human thyrocyte assay was 100 % and 85 %, respectively. The thyroid stimulating activity of GD1 and GD2 (1.5 mg/ml) was equivalent to that of 10 mU/ml of TSH, respectively.

Figure 11 shows the primary structure of a TSH receptor according to the invention, in which a plurality of letters

at any one site indicates the presence of one of the given amino acid residues at that site.

Figure 12 illustrates the cDNA sequence of the cloned human TSH receptor.

The invention relates to a process for the preparation of a recombinant polypeptide possessing thyrotropin receptor activity, which polypeptide comprises an amino-acid sequence represented by the following general formula:

$$[X]_n - [Y]_m - [Z]_p$$

wherein n = 0 or 1; m = 1; p = 0 or 1;
and X, Y and Z are defined as explained below;
the process comprising the expression of a nucleic acid coding for the polypeptide in a host cell transformed by a vector in which the said nucleic acid has been operationally inserted, and which polypeptide, in the case of p = 1, further is characterized in that it is associated with such a host cell, the receptor thus being present in a non-thyroidal cellular environment, or with a membrane preparation which is free of impurities associated with detergent-solubilized thyroid membrane preparations.

The invention further relates to nucleotides to be used in the process of the invention and to certain preferred uses of the polypeptide preparations obtained by the process of the invention.

By "TSH-receptor activity" is meant either TSH-binding properties or anti-TSH receptor antibody-binding properties or the ability to activate adenylyl cycase or phospholipase C via G proteins when exposed to TSH or anti-TSHr antibodies. These properties are easily verified by contacting the polypeptide with for example labelled TSH or labelled anti-TSHr antibodies or by monitoring the adenylyl cyclase activity of a membrane preparation containing the polypeptide. The polypeptides of the invention include the entire TSH receptor as identified by the inventors, and fragments or variants of this polypeptide as defined below. The entire TSH receptor is composed of a signal peptide (20 residues) followed by a large putative extracellular domain (398 residues) containing 5 sites for N-glycosylation, connected to a 346 residue COOH domain containing seven putative transmembrane segments. The amino-acid sequence of the complete receptor is illustrated in fig. 11.

More particularly, the invention relates to a process for the preparation of a recombinant polypeptide possessing thyrotropin receptor activity, which polypeptide i) is characterised in that it comprises an amino-acid sequence represented by the following general formula:

$$[X]_n - [Y]_m - [Z]_p$$

wherein n = 0 or 1; m = 1; p = 0 or 1;
and X, Y and Z are defined as follows (using the one-letter amino-acid symbol and wherein a plurality of letters at any one site indicates the presence of any one of the given amino-acid residues at that site):

X = MRPADLLQLVLLLDLPRDL
PP H A A S

Y = at least the minimum number of consecutive amino-acids of the following sequence necessary for the presentation of immunogenic properties:

```
GGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLI
  K  P          D           H    T          F

ETHLRTIPSHAFSNLPNISRIYVSIDLTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPD
  Q K     R              L    A    R              M          S  S

ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETL
                      GV    V        V               A    A

TLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTA
             I   H                        SA                T      Y

LPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGILESLM

CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQE
      IR           T   G FD       Y     HA    DN Q     DS S

DEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
   L                              V  GN
```

and Z = [I - II -II_i - III - III_i - IV - V - VI - VII - VII_i] wherein the amino-acid sequences I - II - II_i - III - III_i - IV - V - VI - VII - VII_i are independently present or absent and have the following meanings:

```
I = IMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYK
                                      IV
```

or at least 12 consecutive amino-acid residues of this sequence;

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II = LNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSEYYNHA
       T                  I I          IH K Q H Y
```

or at least 12 consecutive amino-acid residues of this sequence;

```
II_i = IDWQTGPGC
                A
```

or at least 2 consecutive amino-acid residues of this sequence;

```
III = NTAGFFTVFASELSVYTLTVITL
      DA
```

or at least 22 consecutive amino-acid residues of this sequence

```
III_i = ERWYAITFAMRLD
          HT  H  Q
```

or at least 2 consecutive amino-acid residues of this sequence;

IV = RKIRLRHACAIMVGGWVCCFLLALLPLVGISSYAKVSICL
C VQ YSV M IFA AA F IF M
A

or at least 12 consecutive amino-acid residues of this sequence;

V = PMDTETPLALAYIVFVLTLNIVAFVIVCCCYVKIYITVRN
IDS SQL VIL L VL I S
MSL V

or at least 12 consecutive amino-acid residues of this sequence;

VI = PQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLIT
M LM

or at least 12 consecutive amino-acid residues of this sequence;

VII = VSNSKILLVLFYPLNSCANPFLYAIFTKAFQRD
T

or at least 12 consecutive amino-acid residues of this sequence;

VII_i =

VFILLSKFGICKRQAQAYRGQRVPPKNSTDIQVQKVTHDMRQGLHNMEDVYELIENS
S AG I R S P Q E L

HLTPKKQGQISEEYMQTVL
N K N

or at least 12 consecutive amino-acid residues of this sequence;

it being understood that any of the above-specified amino-acids can be replaced or deleted, and that extra amino-acid residues may be inserted provided the thyrotropin receptor activity is maintained,
and which polypeptide, ii) in the case of p = 1, further is characterized in that it is associated with a membrane preparation which is free of impurities associated with detergent-solubilized thyroid membrane preparations,
by the expression of a nucleic acid coding for the polypeptide in a host cell transformed by a vector in which the said nucleic acid has been operationally inserted.

The sequence represented by $[x]_n$ in the above general formula corresponds to the signal sequence of the TSH receptor. This part of the polypeptide naturally ensures the transport to the cell membrane of the adjoining [y] and/or [z] fragments, after its production in the cell. Clearly the signal sequence does not need to be present in the polypeptide in cases where transport to the membrane is not required (for example in in vitro translation of the mRNA encoding the polypeptide), or may be replaced by other signal sequences to facilitate production of the recombinant receptor in certain host cells.

The sequence represented by $[z]_p$ in the above general formula corresponds to the COOH domain of the entire polypeptide containing the seven putative transmembrane fragments I-VII, which show homology with the correspond-

ing region of other G-protein coupled receptors. The polypeptides of the invention include, as indicated above, variants of the basic TSH receptor sequence lacking part or all of the transmembrane domain. It is thought that these types of variants may exist naturally as a result of an alternative splicing phenomenon. By homology with other, known G-protein coupled receptors, the seven putative transmembrane segments have tentatively been identified as shown in Fig. 11 (numbered I to VII). The variant polypeptides of the invention include polypeptides missing some or all of the fragments I - VII_i as defined above, which definition includes the putative extracellular and intracellular "loops" occuring between the transmembrane segments (see fig. 6). The transmembrane segment(s) missing may therefore, for example, be a segment selected from segments I to VII as indicated in fig. 11 or may be part of one of those segments, or may be a transmembrane segment in conjunction with its adjoining intracellular and/or extracellular loop.

It is also within the terms of the invention to replace some or all of the transmembrane domain by the transmembrane domain, or part of this domain, of a different receptor, thus giving rise to a hybrid receptor. This type of receptor could be particularly interesting in cases where the extracellular part of the TSH receptor needs to be anchored in a cell membrane having characteristics which are different from, or even incompatible with, the transmembrane portion of the TSH receptor. It is also possible to use as the transmembrane domain in a hybrid receptor any amino-acid sequence exhibiting suitable anchoring properties. Such a sequence could be entirely synthetic or based on any transmembrane protein.

It is to be noted that the invention also embraces the preparation of polypeptides having thyrotropin receptor activity which lack the entire transmembrane domain. In this case, the polypeptide corresponds to the extracellular domain of the naturally occuring receptor. This extracellular part of the receptor which is apparently responsible for ligand binding, is identified by the region [y] in the general formula. A polypeptide lacking the entire transmembrane domain is resp-resented by the general formula $[y]_m$, where m = 1, the [z] part of the sequence being absent. This extracellular part of the receptor [y], is characterised by an imperfect repeat structure which can be aligned as shown in Fig 7. The polypeptides of the invention include variants in which one or more of these repeats is missing. It is however important that sufficient aminoacids be present to allow formation of antibodies (monoclonal or polyclonal). Such immunogenic amino-acid sequences may comprise for example 5, 6, 7, 8 or 9 consecutive amino-acids of the "y" sequence defined above.

In particular, the invention encompasses the preparation of polypeptides in which the second repeat (marked by an arrow in fig 7) is missing.

The invention also relates to nucleic acid sequences coding for the polypeptides of the invention as well as the corresponding complementary sequences. Examples of such sequences are those shown in figs. 5 and 12, and fragments of these sequences, as well as corresponding degenerate sequences. The nucleic acid fragments embraced by the invention normally have at least 8 nucleotides and have preferably at least 12 or preferably at least 16 nucleotides. Such fragments, .or their complementary sequences can be used as primers in the amplification of segments of DNA using the polymerase chain reaction, for example in the production of cDNA coding for the polypeptides having thyrotropin receptor activity.

The process of the invention can be conducted in several different ways. For example, a host cell such as COS 7 cells, CHO cells, NIH3T3 cells, Xenopus oocytes or Y1 cells can be transformed by a vector containing a nucleic acid insert coding for the desired peptide, in conjunction with all the necessary regulatory elements such as promoter, transcription termination signals etc, or microinjected with recombinant mRNA transcribed from appropriate vectors containing the receptor encoded sequence . Expression of the insert normally leads to the insertion of the recombinant polypeptide in the membrane of the cell used as host. In this way, the receptor polypeptide can be used in this form, the receptor thus being present in a cellular environment, or in a solubilised membrane fragment form.

Furthermore, in the case where only a fragment of the polypeptide is required, the correspondingly shorter nucleic acid sequence can be used to transform a suitable host cell, for example, a DNA coding for the putative extracellular region only, or one or more repeats of the repetitive portion of this region.

The invention also makes available antibodies, both polyclonal and monoclonal, to the thyrotropin-receptor polypeptides. As mentioned earlier, in man the TSH-receptor may be the target of autoimune reactions giving rise to hyper- or hypo-stimulation of the thyroid gland by stimulating and blocking autoantibodies respectively. The antigenic nature of the polypeptides of the invention, particularly the putative extracellular domain, permits the preparation of antibodies, which can be used in a variety of studies and assays. The TSH-receptor binds both TSH and anti-TSHr antibodies, thus it is possible in certain studies to replace TSH by anti-TSHr antibodies. The phenomenon of competition between labelled and unlabelled species is particularly useful in such assays.

One such assay falling within the terms of the invention is a process for the quantitative detection of thyrotropine (TSH) or of anti-thyrotropine receptor antibodies (anti-TSHr) in a biological sample wherein a polypeptide obtained according to the process of the invention is contacted with the biological sample suspected of containing TSH or anti-TSHr antibodies and, either simultaneously or subsequently, contacted with labelled TSH, or with labelled anti-TSHr antibodies and the remaining, bound labelled TSH or bound labelled anti-TSHr antibodies after competition between the labelled and unlabelled species, is measured.

In this type of assay, the competition between the labelled TSH or labelled antibodies with the unlabelled TSH or antibodies present in the biological sample is measured as an indication of the concentration of that species in the sample.

Alternatively, instead of using competition between two like-species to measure TSH, it is also possible to use a receptor polypeptide to bind the TSH in the biological sample, and then after washing to add labelled anti-TSH antibodies which selectively detect the bound TSH. This type of assay can also be carried out using immobilized or solubilised receptor polypeptide to bind the anti-TSHr-antibody in a biological sample, followed by detection of the bound antibody by labelled anti-immunoglobulins or protein A or protein G or any other agent capable of recognizing an antibody.

Another means of assaying the TSH or anti-TSHr antibodies in a sample exploits the effect which the binding of these species with the TSH receptor has on the adenylyl cyclase activity of the cell bearing the receptor. Thus, this aspect of the inventions relates to a process for the quantitative detection of TSH or of anti-TSHr antibodies by contacting intact cells operationally transformed by a nucleotide sequence, encoding a polypeptide of the invention or membrane preparations of such cells with the biological' sample suspected of containing TSH or anti-TSHr antibodies and measuring in the intact cells or membranes the change in adenylyl cyclase activity, for example by measuring C-AMP generation or release.

The binding of TSH itself or of stimulating anti-TSHr antibodies to the receptor polypeptide leads to an increase in adenylyl cyclase activity, whereas the binding of blocking anti-TSHr antibodies leads to an inhibition of TSH-induced adenylyl cyclase stimulation. By comparing the adenylyl cyclase activity induced by exposure of the receptor polypeptide to TSH with that induced by antibodies in a sample, it is possible, according to the invention, to distinguish blocking antibodies from stimulating antibodies. In order to quantitatively determine blocking antibodies in a sample, the sample is contacted with the receptor polypeptides either at the same time as with TSH, or before contacting with TSH. In this way the adenylyl cyclase stimulating effect of TSH on the receptor is blocked by the blocking antibodies and is quantified to indicate the concentration of blocking antibodies present in the sample. Such measurements can be carried out in intact cells bearing the TSH receptors of the invention, or in membrane preparations of such cells. Other effector systems which can be used in this type of detection are, for example, activities of phospholiphase C, protein tyrosine kinase, phospholipase A2 etc.

The labels used in the different assays mentioned are those conventionally used in the art, for example, radioactive labelling, enzymatic labelling, labelled anti-immunoglobulins, protein A, protein G, depending upon the type of assay.

Another aspect of the invention relates to a process for the quantitative detection of fragments of TSH receptor in a biological fluid. Such fragments may be found circulating in patients suffering from thyroid disorders. This aspect of the invention involves contacting the sample with antibodies raised against the recombinant polypeptides which have previously been labelled, if necessary, and determining the binding, if any, in the sample by any method involving separation of bound labelled antibody from unboud labelled antibody or by competition between the said fragments and a polypeptide according to the invention. In this latter case it is necessary to label the receptor polypeptide, for example $_{\text{with}}\ ^{125}\text{I}$.

Said antibodies may also be used in the immunohistochemical detection of TSH receptors, for example in endocrinological investigations or in anatomopathology. In this type of process, the antibodies are again labelled to permit their detection.

The polypeptides prepared by the process of the invention may also be used in the purification of stimulating or blocking antibodies to TSHr and of TSH by contacting the polypeptide with a source of TSH or anti-TSHr antibodies, separating the bound and free fractions and finally dissociating the receptor-bound entity. If necessary, further successive purification steps known per se may be added. Such a purification process is facilitated by the immobilisation of the receptor polypeptide, for example in a column or any other solid support.

On the basis of the present invention, there can be designed kits suitable for the detection of TSH or anti-TSHr antibodies. Such kits are characterised in that they contain:

a) a polypeptide as defined above, said polypeptide having thyrotropin receptor activity and being either in an immobilised or solibilised form ;

b) at least one of the following reagents:

i) labelled TSH
ii) labelled anti-TSHr antibodies
iii) reagents necessary for the measurement of adenylyl cyclase activity.

For example, a kit for effecting the detection of autoantibodies directed against the TSH receptor by competition would include the polypeptide prepared by the process of the invention, in immobilised or solubilised form, with labelled TSH or unlabelled TSH in combination with agents permitting the TSH to be labelled. Alternatively, such a kit might

include antibodies to the TSH receptor and means of labelling them, instead of the TSH.

The invention will be illustrated by the following examples:

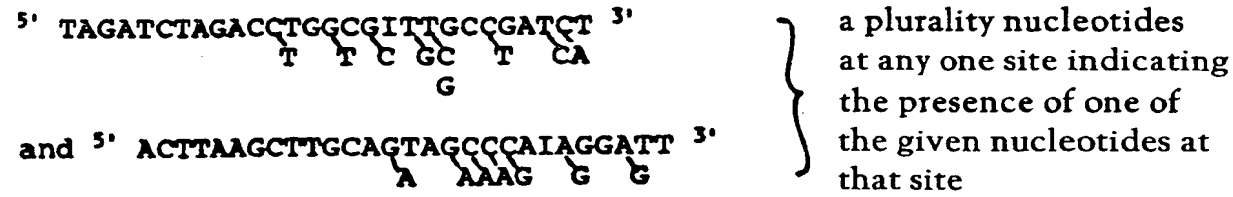
Examples

I - Cloning of dog TSHr

a) Identification of HGMP09

As most G protein-coupled receptor genes do not contain introns in their coding sequence, a similar strategy to that previously described (6) was used, but using different sets of degenerated primers and with human genomic DNA as starting material. Eleven clones displaying sequence similarity with G-protein coupled receptors where thus obtained (7). Out of these, one clone (HGMP09) which was amplified with primers corresponding to transmembrane segments II and VII, presented sequence characteristics suggesting that it belonged to a distinct subfamily of receptors.

The primers used in this amplification were:



A dendrogram constructed from the alignment shown in fig. 1 demonstrated that it is equally distant from all receptors cloned to date (7); in particular, it does not contain the canonical Asp Arg Tyr (DRY) tripeptide close to transmembrane segment III [8] and lacks the Asp residue implicated in the binding of charged amines is adrenergic (Asp113), muscarinic, dopaminergic and serotonergic receptors (9).

b) Identification of dog TSHr

In the frame of a systematic screening for the expression of the new receptors in thyroid tissue, HGMP09 was used as a probe both in Northern blotting experiments with thyroid and non-thyroid tissues, and in screening of a dog thyroid cDNA library. HGMP09 did not hybridize to thyroid mRNA but identified a major 2.6 kb transcript in the ovary and the testis. However, under moderate conditions of stringency it hybridized to one out of 50,000 thyroid cDNA clones suggesting cross-hybridization with a relatively abundant putative receptor of the thyroid. From these characteristics, it was hypothesized that HGMP09 encoded a receptor fragment, distinct from the TSH receptor, but with sequence characteristics expected from close relatives like LH or FSH receptors. A full-length cross-hybridizing clone (dTSHr) was isolated and used as a probe in Northern blots of ten different dog tissues. It hybridized to a 4.9 kb transcript present only in the thyroid gland and in cultured thyrocytes. Interestingly, the signal was much stronger in cultured thyrocytes exposed for several days to the cAMP agonist forskolin than in thyroid tissue. This is a characteristic one would expect from the TSH receptor whose expression is known to be up-regulated by cAMP agonists in cultured cells (10). A 4,417 bp cDNA clone was sequenced completely. It contains an open reading frame of 764 aminoacids beginning with a 20 residue signal peptide, as predicted by Von Heijne algorithm (11) (fig.2a). Comparison to known G-protein coupled receptors (see hereunder and fig. 2b) and hydropathy profile analysis (not shown) demonstrated a 346 residue C-terminal structure with seven putative transmembrane domains preceded by 398 aminoacids constituting a large putative extracellular domain.

c) Expression of dog TSHr

The encoded polypeptide was unambiguously identified as the TSH receptor by expression of the cDNA in a variety of systems. Microinjection of recombinant mRNA in adrenocortical Y1 cells and in Xenopus oocytes conferred a TSH responsive phenotype to both systems. Y1 cells responded to TSH by a characteristic morphological change which is triggered by elevation of cAMP in the cytoplasm (12,13). Xenopus oocytes (fig. 3) displayed a dose-dependant increase in cAMP which was specific for stimulation by TSH and corresponded to the expected sensivity of the dog receptor to bovine TSH (half-maximal effect around 0.3 nM) (14). Transient expression of the receptor cDNA was obtained in Cos7 cells (fig 4). Specific binding of ^{125}I TSH to membranes was observed only in transfected cells. The displacement curve

of the label by TSH presented characteristics very similar to that obtained with membranes from dog thyrocytes (half-maximal displacement at 0.4 nM and 0.16 nM for cos cells and thyrocytes, respectively) (fig. 4c). The slight rightward shift of the displacement curve obtained with Cos7 cell membranes may reflect the higher amount of receptors in this system.

The cDNA coding for the dog TSH receptor was sequenced completely. The sequence is given in fig. 5.

d) Comparison of TSHr with LH-CGr

Comparison of the TSH receptor with the LH-CG receptor cloned recently (15, 16) reveals interesting common characteristics which make them members of a new subfamily of G-protein coupled receptors. They both display a long aminoterminal extension containing multiple sites for N glycosylation (five in the TSH receptor). The TSH receptor has an extra 52 residue insert close to the junction between the putative extracellular domain and the first transmembrane segment (fig. 2a). The overall sequence similarity between the extracellular domains of the TSH and LH-CG receptors is 45% (fig 2a). The similarity between a segment of soybean lectin and the rat LH receptor (15) is not conserved in the TSH receptor, which suggests that it may be fortuitous. The C-terminal half of the TSH receptor containing the transmembrane segments is 70% similar to both the pig and rat LH receptors (fig. 2a). The homology is particularly impressive in the transmembrane segments themselves, where stretches of up to 24 identical residues are observed in a row (transmembrane region III). Also, the carboxyl terminal region of the third putative intracellular loop, which is particularly short in TSH and LH receptors and which has been implicated in the interaction with $G_{\alpha s}$, (8, 9), is identical in both receptor types. This pattern of similarities gives support to the view that the extracellular domain would be involved in the recognition of the ligands [4], while the membrane-inserted domain would be responsible for the activation of $G_{\alpha s}$ (15, 16). Together, the TSH and LH-CG receptors, and HGMP09 (there is strong preliminary evidence that HGMP09 could actually be the FSH receptor (7)) constitute clearly a distinct subfamily of G-protein coupled receptors. A sequence similarity dendrogram (17) including most of the G-protein coupled receptors cloned to date demonstrates both their close relationships and their distance from the bulk of the other receptors (fig. 2b). The complete sequence of the FSH receptor will reveal whether the known ability of LH-CG to stimulate the TSH receptor (18) is reflected by a higher sequence similarity of the extracellular domains of LH and TSH receptors.

e) Identification of a dog TSHr variant

Screening of the dog thyroid cDNA library (30) with the HGMP09 clone (thought to be part of the FSH receptor), gave rise to 16 positive clones out of the 840,000 screened plaques. Hybridization was carried out at 42°C in 35% formamide and the filters were washed at 65°C in 2 x SSC, 0.1% SDS before autoradiography. 12 clones were purified to homogeneity and analyzed by EcoRI digestion. Three clones (dTSHR1, dTSHR2 and dTSHR3) were subcloned in M13mp18 and pBS vectors. dTSHR1 and dTSHR2 consisted of two EcoRI fragments of respectively 2800 and 1500 bp. dTSHR3 was shorter, and consisted of 2200 and 1500 bp EcoRI fragments. Restriction analysis of the 2800 bp fragments of dTSHR1 and dTSHR2 revealed slight differences in the restriction map, the main discordance being the presence of a PstI restriction site in dTSHR1 and its absence in dTSGR2. dTSHR1 was sequenced completely and revealed an open reading frame of 764 codons which was identified as the thyrotropin receptor by expression of the cDNA in oocytes and cell cultures (see example I(b) + fig 5) . dTSHR3 was shown by sequencing to be completely colinear with dTSHR1 but his clone lacked 600 bp at its 5' end. Because of the difference in the restriction map of dTSHR1 and dTSHR2, this latter clone was also sequenced on both strands.

The sequence revealed a number of mutations when compared with the dTSHR1 clone. A total of 5 mutations, including tvo single base substitutions, one single base deletion, one single base insertion and one 5 base insertion were found scattered in the 2060 bp long 3' untranslated region (not shown). However, the main difference between dTSHR2 and dTSHR1 was located in the coding region, and consisted in a 75 bp deletion located 240 bp after the start codon. The corresponding 25 amino-acids deletion in the protein itself is located in the long NH2 terminal extracellular domain which is characteristic of the TSH receptor (25) and its recently cloned close relative, the LH receptor (15, 16) (fig. 6). As in the LH receptor, the NH2 terminal part of the thyrotropin receptor is characterized by an imperfect repeat structure that can be aligned as indicated in fig. 7. These repeats are similar in structure to the leucine-rich repeats found in the various proteins comprising the family of leucine-rich glycoproteins (26, 15), and references therein). The deletion in the dTSHR2 clone corresponds exactly to one of these repeats, in a region of the protein where the repeat length is regular and their alignment unambiguous. The existence of such variant reinforces considerably the significance of this repeated structure and sets up interesting questions concerning its functional meaning and the structure of the chromosomal gene.

The extracellular domains of TSH and LH receptors are apparently responsible for the ligand binding (4). The deleted repeat also contains one of the 5 consensus sequences for N-glycosylation. Glycosylation of the TSH receptor could be important for ligand binding or signal transduction. If, and to what extent, the lack of this repeat influences

the binding capabilities and/or the function of the receptor variant, is not yet known. Comparison of cell lines expressing this variant with the presently available stable transfectants expressing the full size receptor should partially answer this question. The functional analysis of other in-vitro generated mutants of the TSH receptor will complete the study.

The deletion of a full repeat gives also some insight on the structure of the TSH receptor gene. It is highly probable that the repeat unit corresponds to a complete exon, and it is therefore possible that all repeats would be separated by introns. It is interesting to note that most of the genes coding for G-protein coupled receptors are completely devoid of intronic structures. The functional or evolutionary significance of this observation is not known, but a highly fragmented exonic structure of the glycoprotein hormone receptor genes would be in clear contrast to the rest of the receptor family.

II - Cloning of the human TSHr

A human lambda gt11 cDNA library (29) was screened with a fragment of the dog TSHr (25). Out of the 218 clones scored as positive (1/6000), 24 were plaque-purified to homogeneity and the size of the inserts was determined. Two clones which harbored inserts of 2370 bp and 3050 bp, respectively, Were subcloned as overlapping fragments in M13 derivatives and sequenced (fig 12). A total of 4272 bp were determined in which a 2292 bp open reading frame was identified. When translated into protein, the coding sequence showed an overall 90.3 % similarity with the dog TSHr (Fig. 8) [1]. It displayed all the characteristics described recently for the subfamily of G protein-coupled receptors binding glycoprotein hormones (25, 15, 16); a signal peptide (20 residues) followed by a large putative extracellular domain (398 residues) containing 5 sites for N-glycosylation, connected to a 346 residue carboxyl-terminal domain containing seven putative transmembrane segments (fig. 8). It has been suggested that the amino-terminal domain, which is not found in other G protein-coupled receptors, might correspond to the region involved in the binding of the bulky pituitary and placental glycoprotein hormones (25, 15, 16).

Variants of the hTSHr

When probed with the putative human TSHr, a Northern blot of RNA from human placenta, testis and thyroid revealeld two major 4.6 and 4.4 kb thyroid-specific transcrips. Minor thyroid-specific RNA species of smaller size were also observed. Although the former could simply correspond to multiple polyadenylation sites in the 3' region of the gene, this situation is reminiscent of what has been observed for the porcine LH-CG receptor. In this case, multiple transcripts were found to correspond to variants of the receptor cDNA lacking the potential to encode the membrane spanning segments (16). Whether this observation with important implications on receptor function and regulation also applies to the human TSHr will await sequencing of additional clones from the cDNA library.

Expression of hTSHr

To provide definite proof that the clones isolated encoded a human TSH receptor, the coding sequence was inserted in the SV40-based vector pSVL, and the resulting construct transfected in Cos-7 cells (24). Membranes prepared from transfected cells demonstrated specific binding of [^{125}I]TSH (fig. 9). The unlabelled competitor TSH was bovine. The characteristics of the displacement curve with unlabelled TSH were similar to those observed with the dog TSHr assayed under similar conditions (half maximal displacement around 0.5 nM) (25).

From the sequence similarity with dog TSHr, the tissue specific expression of the corresponding transcripts and the binding studies on membranes from transfected COS-7 cells, it was concluded that a **bona fide** human TSHr has been cloned.

Antibodies to hTSHr

To investigate the relevance of the cloned human TSHr to thyroid autoimmunity, competition was tested between [^{125}I]TSH and immunoglobulins prepared from patients, for binding to the recombinant receptor expressed in Cos-7 cells (fig 10). Immunoglobulins were prepared from the serum of patients or normal individuals by ammonium sulphate precipitation. They were dissolved in water and dialysed extensively against Dulbecco's modified Eagle medium. While immunoglobulins from normal individuals did not displace [^{125}I]TSH, samples from two patients with idiopathic myxoedema clearly did, in a dose-dependant manner. The steep dose-response which was observed suggests that immunoglobulins from these patients present a very high affinity for the recombinant receptor. When samples from two patients with Graves' disease having high levels of thyroid stimulating immunoglobulins in the circulation were tested, one of them showed limited ability to displace labelled TSH under the conditions of the assay (fig.10). The difference in the potency of these two categories of immunoglobulins to displace TSH from the receptor expressed in Cos-7 cells may reflect differences in their affinity for a common antigen. Alternatively, despite previous studies suggesting that

both stimulating and blocking antibodies would bind to the same part of the TSHr (26, 27), it may correspond to more basic differences in the actual nature of their respective antigenic targets. Studies where binding activity of a larger collection of immunoglobulins are compared to their ability to activate adenylate cyclase in permanently transfected cells will help to clarify this point.

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Claims

1. A process for the preparation of a recombinant polypeptide possessing thyrotropin receptor activity, which polypeptide i) is characterised in that it comprises an amino-acid sequence represented by the following general formula:

$$[X]_n - [Y]_m - [Z]_p$$

wherein n = 0 or 1; m = 1; p = 0 or 1;
and X, Y and Z are defined as follows (using the one-letter amino-acid symbol and wherein a plurality of letters at any one site indicates the presence of any one of the given amino-acid residues at that site):

```
X = MRPADLLQLVLLLDLPRDL
      PP  H A   A   S
```

Y = at least the minimum number of consecutive amino-acids of the following sequence necessary for the presentation of immunogenic properties:

GGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLI
K P D H T F

ETHLRTIPSHAFSNLPNISRIYVSIDLTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPD
Q K R L A R M S S

ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETL
GV V V A A

TLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTA
I H SA T Y

LPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGILESLM

CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQE
IR T G FD Y HA DN Q DS S

DEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
L V GN

and Z = [I - II -II_i - III - III_i - IV - V - VI - VII - VII_i] wherein the amino-acid sequences I - II - II_i - III - III_i - IV - V - VI - VII - VII_i are independently present or absent and have the following meanings:

I = IMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYK
IV

or at least 12 consecutive amino-acid residues of this sequence;

II = LNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSEYYNHA
T I I IH K Q H Y

or at least 12 consecutive amino-acid residues of this sequence;

II_i = IDWQTGPGC
A

or at least 2 consecutive amino-acid residues of this sequence;

III = NTAGFFTVFASELSVYTLTVITL
DA

or at least 22 consecutive amino-acid residues of this sequence

III_i = ERWYAITFAMRLD
HT H Q

or at least 2 consecutive amino-acid residues of this sequence;

IV = RKIRLRHACAIMVGGWVCCFLLALLPLVGISSYAKVSICL
C VQ YSV M IFA AA F IF M
A

or at least 12 consecutive amino-acid residues of this sequence;

V = PMDTETPLALAYIVFVLTLNIVAFVIVCCCYVKIYITVRN
IDS SQL VIL L VL I S
MSL V

or at least 12 consecutive amino-acid residues of this sequence;

VI = PQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLIT
M LM

or at least 12 consecutive amino-acid residues of this sequence;

VII = VSNSKILLVLFYPLNSCANPFLYAIFTKAFQRD
T

or at least 12 consecutive amino-acid residues of this sequence;

VII_i =

VFILLSKFGICKRQAQAYRGQRVPPKNSTDIQVQKVTHDMRQGLHNMEDVYELIENS
S AG I R S P Q E L

HLTPKKQGQISEEYMQTVL
N K N

or at least 12 consecutive amino-acid residues of this sequence;

it being understood that any of the above-specified amino-acids can be replaced or deleted, and that extra amino-acid residues may be inserted provided the thyrotropin receptor activity is maintained,
by the expression of a nucleic acid coding for the polypeptide in a host cell transformed by a vector in which said nucleic acid has been operationally inserted,
and which polypeptide, ii) in the case of p = 1, further is characterized in that it is associated with such a host cell, the receptor thus being present in a non-thyroidal cellular environment, or with a membrane preparation which is free of impurities associated with detergent-solubilized thyroid membrane preparations.

2. Process according to claim 1, characterised in that the polypeptide "Y" is composed of at least one of the following sub-sequences: Y_1 to Y_{13}:

Y_1: GMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQT
K P D H T

Y_2: LKLIETHLRTIPSHAFSNLPNISR
F Q K R

Y_3: IYVSIDLTLQQLESHSFYNLSKVTH
L A R M

Y_4: IEIRNTRNLTYIDPDALKELPLLKF
S S

Y_5: LGIFNTGLKMFPDLTKVYSTDIFFI
GV V V

Y_6: LEITDNPYMTSIPVNAFQGLCNETL
A A

Y_7: TLKLYNNGFTSVQGYAFNGTKLDAV
I H

Y_8: YLNKNKYLTVIDKDAFGGVYSGPS
SA T

Y_9: LLDVSQTSVTALPSKGLEHLKELIA
Y

Y_{10}: RNTWTLKKLPLSLSFLHLTRADL

Y_{11}: SYPSHCCAFKNQKKIRGILESLMCN

Y_{12}: ESSMQSLRQRKSVNALNSPLHQEYE
IR T G FD

Y_{13}:

ENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQEDEIIGFGQELKNPQEETLQAFDSH
Y HA DN Q DS S L

YDYTICGDSEDMVCTPKSDEFNPCED
V GN.

3. Process according to any of claims 1 or 2, in which the part of the polypeptide forming the transmembrane domain [Z] is heterologous to the TSH receptor, the polypeptide thus being a hybrid polypeptide.

4. Process according to claim 1, wherein the polypeptide is characterised by the sequence shown in fig. 11.

5. Nucleotide sequences coding for the polypeptide to be prepared according to the process of claims 1 to 4, and nucleotide sequences which are complementary to said sequences, provided that said nucleotide sequence is not the sequence contained in the clone tr.12.6-1 deposited under ATCC accession number 40651.

6. Nucleotide sequence according to claim 5, characterised in that the sequence is a DNA sequence having the sequence shown in fig. 5 or fig 12 or a sequence complementary to said sequences.

7. Use of intact cells obtained in a process according to claims 1 to 4, provided that p=1, in a process for the quantitative detection of TSH or of anti-TSHr antibodies, wherein said intact cells are contacted with the biological sample suspected of containing TSH or anti-TSHr antibodies and wherein in said intact cells the change in adenylyl cyclase activity is measured, for example by measuring c-AMP generation or release.

8. Use of intact cells obtained in a process in accordance with claim 1 to 4 according to claim 7, wherein the intact cells expressing the receptor polypeptide are contacted with the biological sample and, either simultaneously or subsequently with TSH, thereby allowing any inhibition of the adenylyl cyclase activating effect of TSH by "blocking" anti-TSHr antibodies present in the biological sample to be detected.

Patentansprüche

1. Ein Verfahren zur Herstellung eines rekombinanten Polypeptids, das Thyrotropinrezeptor-Aktivität besitzt, wobei das Polypeptid (i) dadurch gekennzeichnet ist, daß es eine Aminosäuresequenz aufweist, die von der folgenden allgemeinen Formel wiedergegeben wird:

$$[X]_n - [Y]_m - [Z]_p$$

worin n = 0 oder 1; m = 1; p = 0 oder 1;
und X, Y und Z wie folgt definiert sind (unter Verwendung des Einbuchstaben-Aminosäuresymbols und wobei eine Vielzahl von Buchstaben in irgendeiner Position die Anwesenheit von irgendeinem der angegebenen Aminosäurereste in dieser Position anzeigt):

X = MRPADLLQLVLLLDLPRDL
PP H A A S

Y = wenigstens die Mindestanzahl von aufeinanderfolgenden Aminosäuren der nachfolgenden Sequenz, die erforderlich ist, daß immunogene Eigenschaften ausgeprägt werden:

```
GGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLI
  K  P          D          H   T         F

ETHLRTIPSHAFSNLPNISRIYVSIDLTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPD
  Q K    R             L   A   R           M          S  S

ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETL
                    GV   V       V           A   A

TLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTA
            I  H                   SA              T     Y

LPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGILESLM

CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQE
     IR          T  G FD     Y    HA   DN Q    DS S

DEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
   L                          V  GN
```

und Z = [I - II -II_i - III - III_i - IV - V - VI - VII - VII_i] worin die Aminosäuresequenzen I - II - II_i - III - III_i - IV - V - VI - VII - VII_i unabhängig voneinander vorhanden sind oder fehlen und die folgenden Bedeutungen aufweisen:

```
I =        IMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYK
                                          IV
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
II =       LNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSEYYNHA
             T                 I I          IH K Q H Y
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
IIi =      IDWQTGPGC
                 A
```

oder wenigstens 2 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
III =      NTAGFFTVFASELSVYTLTVITL
           DA
```

oder wenigstens 22 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
IIIi =     ERWYAITFAMRLD
              HT  H  Q
```

oder wenigstens 2 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
IV =    RKIRLRHACAIMVGGWVCCFLLALLPLVGISSYAKVSICL
        C VQ    YSV   M IFA AA  F IF     M
                A
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
V =     PMDTETPLALAYIVFVLTLNIVAFVIVCCCYVKIYITVRN
          IDS  SQL VIL  L  VL  I   S
                 MSL V
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
VI =    PQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLIT
                                  M             LM
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

```
VII =   VSNSKILLVLFYPLNSCANPFLYAIFTKAFQRD
          T
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

VII_i =

```
VFILLSKFGICKRQAQAYRGQRVPPKNSTDIQVQKVTHDMRQGLHNMEDVYELIENS
                        S    AG  I    R    S P  Q E   L

HLTPKKQGQISEEYMQTVL
   N      K  N
```

oder wenigstens 12 aufeinanderfolgende Aminosäurereste aus dieser Sequenz;

wobei gilt, daß irgendwelche der oben angegebenen Aminosäuren ersetzt werden oder entfallen können, und daß zusätzliche Aminosäurereste eingeschoben werden können, vorausgesetzt, daß die Thyrotropinrezeptor-Aktivität beibehalten wird,

durch Expression einer Nukleinsäure, die für das Polypeptid kodiert, in einer Wirtszelle, die durch einen Vektor transformiert wurde, in den die genannte Nukleinsäure operativ eingesetzt wurde,

und wobei das Polypeptid ii) im Falle von p = 1 außerdem dadurch gekennzeichnet ist, daß es mit einer derartigen Wirtszelle assoziiert ist, so daß der Rezeptor in einer nicht-thyroidalen zellulären Umgebung vorliegt, oder in einer Membranpräparation, die frei von Verunreinigungen ist, die mit Detergens-solubilisierten Schilddrüsenmembranpräparationen assoziiert sind.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Polypeptid "Y" aus wenigstens einer der folgenden

Untersequenzen Y_1 bis Y_{13} zusammengesetzt ist:

Y_1: GMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQT
K P D H T

Y_2: LKLIETHLRTIPSHAFSNLPNISR
F Q K R

Y_3: IYVSIDLTLQQLESHSFYNLSKVTH
L A R M

Y_4: IEIRNTRNLTYIDPDALKELPLLKF
S S

Y_5: LGIFNTGLKMFPDLTKVYSTDIFFI
GV V V

Y_6: LEITDNPYMTSIPVNAFQGLCNETL
A A

Y_7: TLKLYNNGFTSVQGYAFNGTKLDAV
I H

Y_8: YLNKNKYLTVIDKDAFGGVYSGPS
SA T

Y_9: LLDVSQTSVTALPSKGLEHLKELIA
Y

Y_{10}: RNTWTLKKLPLSLSFLHLTRADL

Y_{11}: SYPSHCCAFKNQKKIRGILESLMCN

Y_{12}: ESSMQSLRQRKSVNALNSPLHQEYE
IR T G FD

Y_{13}:

ENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQEDEIIGFGQELKNPQEETLQAFDSH
Y HA DN Q DS S L

YDYTICGDSEDMVCTPKSDEFNPCED
V GN.

3. Verfahren nach irgendeinem der Ansprüche 1 oder 2, bei dem der Teil des Polypeptids, der die Transmembran-Domäne [Z] bildet, zu dem TSH-Rezeptor heterolog ist, so daß das Polypeptid ein Hybrid-Polypeptid ist.

4. Verfahren nach Anspruch 1, bei dem das Polypeptid durch die Sequenz charakterisiert ist, die in Figur 11 gezeigt ist.

5. Nukleotidsequenzen, die für das nach dem Verfahren der Ansprüche 1 bis 4 herzustellende Polypeptid kodieren, sowie Nukleotidsequenzen, die zu diesen Sequenzen komplementär sind, vorausgesetzt, daß die genannte Nukleotidsequenz nicht die Sequenz ist, die in dem Klon tr.12.6-1 enthalten ist, der unter ATCC-Hinterlegungsnummer 40651 hinterlegt ist.

6. Nukleotidsequenz nach Anspruch 5, dadurch gekennzeichnet, daß die Sequenz eine DNA-Sequenz ist, die die Sequenz aufweist, die in Figur 5 oder Figur 12 gezeigt ist, oder eine Sequenz, die zu diesen Sequenzen komplementär ist.

7. Verwendung von intakten Zellen, die nach einem Verfahren gemäß den Ansprüchen 1 bis 4 erhalten werden, wenn p = 1, in einem Verfahren für den quantitativen Nachweis von TSH oder von Anti-TSHr-Antikörpern, wobei die genannten intakten Zellen mit der biologischen Probe in Kontakt gebracht werden, von der vermutet wird, daß sie TSH oder Anti-TSHr-Antikörper enthält, und wobei in den genannten intakten Zellen die Veränderung der Adenylylcyclase-Aktivität gemessen wird, beispielweise durch Messung der c-AMP-Erzeugung oder -Freisetzung.

8. Verwendung von intakten Zellen, die in einem Verfahren gemäß Anspruch 1 bis 4 erhalten werden, gemäß Anspruch 7, wobei die intakten Zellen, die das Rezeptor-Polypeptid exprimieren, mit der biologischen Probe in Kontakt gebracht werden und entweder gleichzeitig oder anschließend mit TSH, wodurch man eine Inhibierung des Adenylylcyclase-aktivierenden Effekts von TSH durch "blockierende" Anti-TSHr-Antikörper ermöglicht, die in der zu untersuchenden biologischen Probe vorhanden sind.

Revendications

1. Procédé de préparation d'un polypeptide recombinant possédant une activité de récepteur de la thyrotropine, lequel polypeptide i) est caractérisé en ce qu'il comprend une séquence d'acides aminés représentée par la formule générale suivante :

$$[X]_n - [Y]_m - [Z]_p$$

dans laquelle n = 0 ou 1, m = 1, p = 0 ou 1,
et X, Y et Z sont définis de la manière suivante (à l'aide des symboles d'acides aminés à une lettre et une multiplicité de lettres à un site quelconque indiquant la présence à ce site de l'un quelconque des résidus d'acides aminés donnés) :

X = MRPADLLQLVLLLDLPRDL
PP H A A S

Y = au moins le nombre minimum d'acides aminés consécutifs de la séquence suivante nécessaire pour la présentation des propriétés immunogènes :

GGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLI
K P D H T F

ETHLRTIPSHAFSNLPNISRIYVSIDLTLQQLESHSFYNLSKVTHIEIR
Q K R L A R M

NTRNLTYIDPD
S S

ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSI
GV V V A

PVNAFQGLCNETL
A

TLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPS
I H SA T

LLDVSQTSVTA
Y

LPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKI

RGILESLM

CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNA
IR T G FD Y HA DN Q DS S

HYYVFFEEQE

DEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
L V GN

et Z = [I - II - II_i - III - III_i - IV - V - VI - VII - VII_i] où les séquences d'acides aminés I - II - II_i - III - III_i - IV - V - VI - VII - VII_i sont indépendamment présentes ou absentes et ont les significations suivantes :

I = IMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYK
IV

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

II = LNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSEYYNHA
T I I IH K Q H Y

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

II_i = IDWQTGPGC
A

ou au moins 2 résidus d'acides aminés consécutifs de cette séquence ;

III = NTAGFFTVFASELSVYTLTVITL
DA

ou au moins 22 résidus d'acides aminés consécutifs de cette séquence ;

III_i = ERWYAITFAMRLD
HT H Q

ou au moins 2 résidus d'acides aminés consécutifs de cette séquence ;

IV = RKIRLRHACAIMVGGWVCCFLLALLPLVGISSYAKVSICL
C VQ YSV M IFA AA F IF M
A

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

V = PMDTETPLALAYIVFVLTLNIVAFVIVCCCYVKIYITVRN
IDS SQL VIL L VL I S
MSL V

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

VI = PQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLIT
M LM

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

VII = VSNSKILLVLFYPLNSCANPFLYAIFTKAFQRD
T

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

VII_i = VFILLSKFGICKRQAQAYRGQRVPPKNSTDIQVQKVTHDMRQ
S AG I R

GLHNMEDVYELIENSHLTPKKQGQISEEYMQTVL
S P Q E L N K N

ou au moins 12 résidus d'acides aminés consécutifs de cette séquence ;

étant entendu que l'un quelconque des acides aminés spécifiés ci-dessus peut être remplacé ou délété et que des résidus d'acides aminés supplémentaires peuvent être insérés à condition que l'activité de récepteur de la thyrotropine soit maintenue, par l'expression d'un acide nucléique codant le polypeptide dans une cellule hôte transformée par un vecteur dans lequel ledit acide nucléique a été inséré de manière active,
et lequel polypeptide, ii) dans le cas où p = 1 et caractérisé encore en ce qu'il est associé à une telle cellule hôte, le récepteur étant ainsi présent dans un environnement cellulaire non thyroïdien, ou à une préparation de membranes qui est dépourvue d'impuretés associées aux préparations de membranes thyroïdiennes stabilisées par des détergents.

2. Procédé selon la revendication 1, caractérisé en ce que, dans le polypeptide, "Y" est composé d'au moins l'une des sous-séquences suivantes :

Y_1 : GMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQT
K P D H T

Y_2 : LKLIETHLRTIPSHAFSNLPNISR
F Q K R

Y_3 : IYVSIDLTLQQLESHSFYNLSKVTH
L A R M

Y_4 : IEIRNTRNLTYIDPDALKELPLLKF
S S

Y_5 : LGIFNTGLKMFPDLTKVYSTDIFFI
GV V V

Y_6 : LEITDNPYMTSIPVNAFQGLCNETL
A A

Y_7 : TLKLYNNGFTSVQGYAFNGTKLDAV
I H

Y_8 : YLNKNKYLTVIDKDAFGGVYSGPS
SA T

Y_9 : LLDVSQTSVTALPSKGLEHLKELIA
Y

Y_{10} : RNTWTLKKLPLSLSFLHLTRADL

Y_{11} : SYPSHCCAFKNQKKIRGILESLMCN

Y_{12} : ESSMQSLRQRKSVNALNSPLHQEYE
IR T G FD

Y_{13} : ENLGDSIVGYKEKSKFQDTHNNAHYYFFEEQEDEIIGFG
Y HA DN Q DS S L

QELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
V GN

3. Procédé selon l'une quelconque des revendications 1 ou 2, dans lequel la partie du polypeptide formant le domaine transmembranaire [Z] est hétérologue au récepteur de la TSH, le polypeptide étant ainsi un polypeptide hybride.

4. Procédé selon la revendication 1, dans lequel le polypeptide est caractérisé par la séquence représentée sur la figure 11.

5. Séquences nucléotidiques codant le polypeptide destiné à être préparé selon le procédé des revendications 1 à 4, et séquences nucléotidiques qui sont complémentaires desdites séquences, à condition que ladite séquence nucléotidique ne soit pas la séquence contenue dans le clone tr.12.6-1 déposé sous le numéro de dépôt ATCC 40651.

6. Séquence nucléotidique selon la revendication 5, caractérisée en ce que la séquence est une séquence d'ADN ayant la séquence représentée sur la figure 5 ou la figure 12 ou une séquence complémentaire desdites séquences.

7. Utilisation de cellules intactes obtenues dans un procédé selon les revendications 1 à 4, à condition que p = 1, dans un procédé de détection quantitative de la TSH ou d'anticorps anti-TSHr, dans laquelle lesdites cellules intactes sont mises en contact avec l'échantillon biologique supposé contenir de la TSH ou des anticorps anti-TSHr et dans laquelle, dans lesdites cellules intactes, la variation d'activité d'adénylate cyclase est mesurée, par exemple par mesure de la production ou de la libération d'AMPc.

8. Utilisation selon la revendication 7 de cellules intactes obtenues dans un procédé selon les revendications 1 à 4, dans laquelle les cellules intactes exprimant le polypeptide du récepteur sont mises en contact avec l'échantillon biologique et, simultanément ou successivement, avec de la TSH, pour permettre ainsi la détection de toute inhibition de l'effet activant l'adénylate cyclase de la TSH par des anticorps anti-TSHr "bloquants" présents dans l'échantillon biologique.

Fig 1

```
              3rd transmembrane segment
HGMP09    CDA AGFFTVFASELSVYTLTAITL ERWHTITHAMQLDCKVQLRHAASVMVMG-WIFAF
          *        *     *   *  *    *   **             *         *   *
M2HUM     CDL WLALDYVVSNASVMNLLIISF DRYFCVTKPLTYPVKRTTKMAGMMIAAA-WVLSF
M3HUM     CDL WLALDYVVSNASVMNLLIISF DRYFCVTKPLTYPARRTTKMAGLMIAAA-WVLSF
M1HUM     CDL WLALDYVASNASVMNLLLISF DRYFSVTRPLSYRAKRTPRRAALMIGLA-WLVSF
M4HUM     CDL WLAIDYVASNASVMNLLVISF DRYFSITRPLTYRAKRTTKRAGVMIGLA-WVISF
A1ADRHAM  CDI WAAVDVLCCTASILSLCAISI DRYIGVRYSLQYPTLVTRRKAILALLSV-WVLST
D2R       CDI FVTLDVMMCTASILNLCAISI DRYTAVAMPMLYNTRYSSKRRVTVMIAIVWVLSF
B1ADRHUM  CEL WTSVDVLCVTASIETLCVIAL DRYLAITSPFRYQSLLTRARARGLVCTV-WAISA
B2ADRHUM  CEF WTSIDVLCVTASIETLCVIAV DRYFAITSPFKYQSLLTKNKARVIILMV-WIVSG
BADRHAM   CEF WTSIDVLCVTASIETLCVIAV DRYIAITSPFKYQSLLTKNKARMVILMV-WIVSG
RDC4      CDI WLSSDITCCTASILHLCVIAL DRYWAITDALEYSKRRTAGRAAVMIATV-WVISI
A2ADRHUM  CEI YLALDVLFCTSSIVHLCAISL DRYWSITQAIEYNLKRTRRRIKAIIITC-WVISA
G21 5HT1A CDL FIALDVLCCTSSILHLCAIAL DRYWAITDPIDYVNKRTP-RPRALISLT-WLIGF
5HT1CRAT  CPV WISLDVLFSTASIMHLCAISL DRYVAIRNPIEHSRFNSRTKAIMKIAIV-WAISI
5HT2R     CAI WIYLDVLFSTASIMHLCAISL DRYVAIQNPIHHSRFNSRTKAFLKIIAV-WTISV
RDC8      CLF FACFVLVLTQSSIFSLLAIAI DRYIAIRIPLRYNGLVTGTRAKGIIAVC-WVLSF
RDC7      CLM VACPVLILTQSSILALLAIAV DRYLRVKIPLRYKTVVTPRRAAVAIAGC-WILSF
SKRBOV    CYF QNLFPITAMFVSIYSMTAIAA DRYMAIVHPFQP--RLSAPGTRAVIAGI-WLVAL
RDC1      CKI THLIFSINLFGSIFFLTCMSV DRYLSITYFASTSSRRKKVVRRAVCVLV-WLLAF
                  ^                 ^ ^
```

2a

Fig

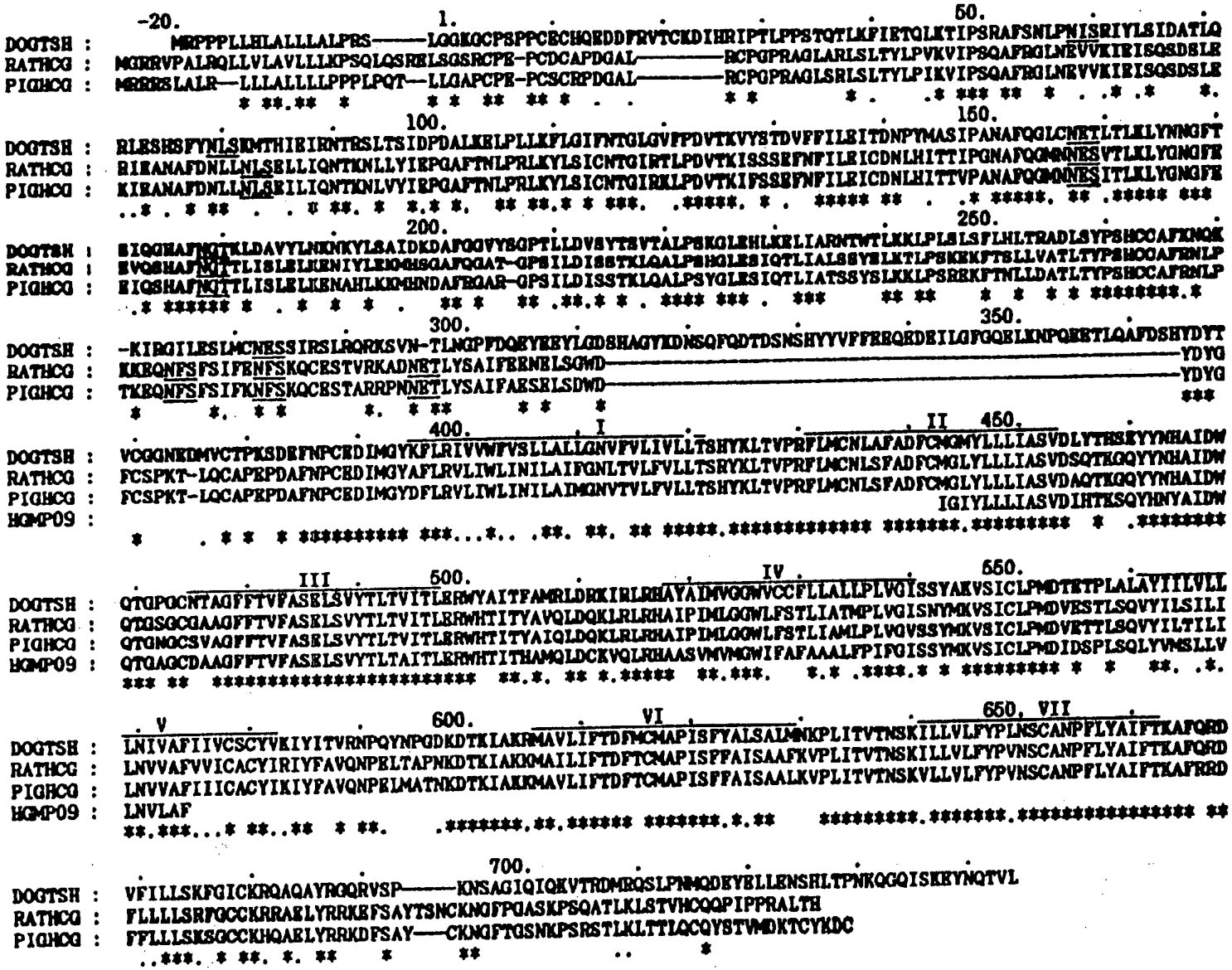

-20.
1.
50.
DOGTSH :
RATHCG :
PIGHCG :
100.
150.
200.
250.
300.
350.
400.
I
II
450.
HGMP09 :
III
500.
IV
550.
V
600.
VI
650.
VII
700.

Fig 26

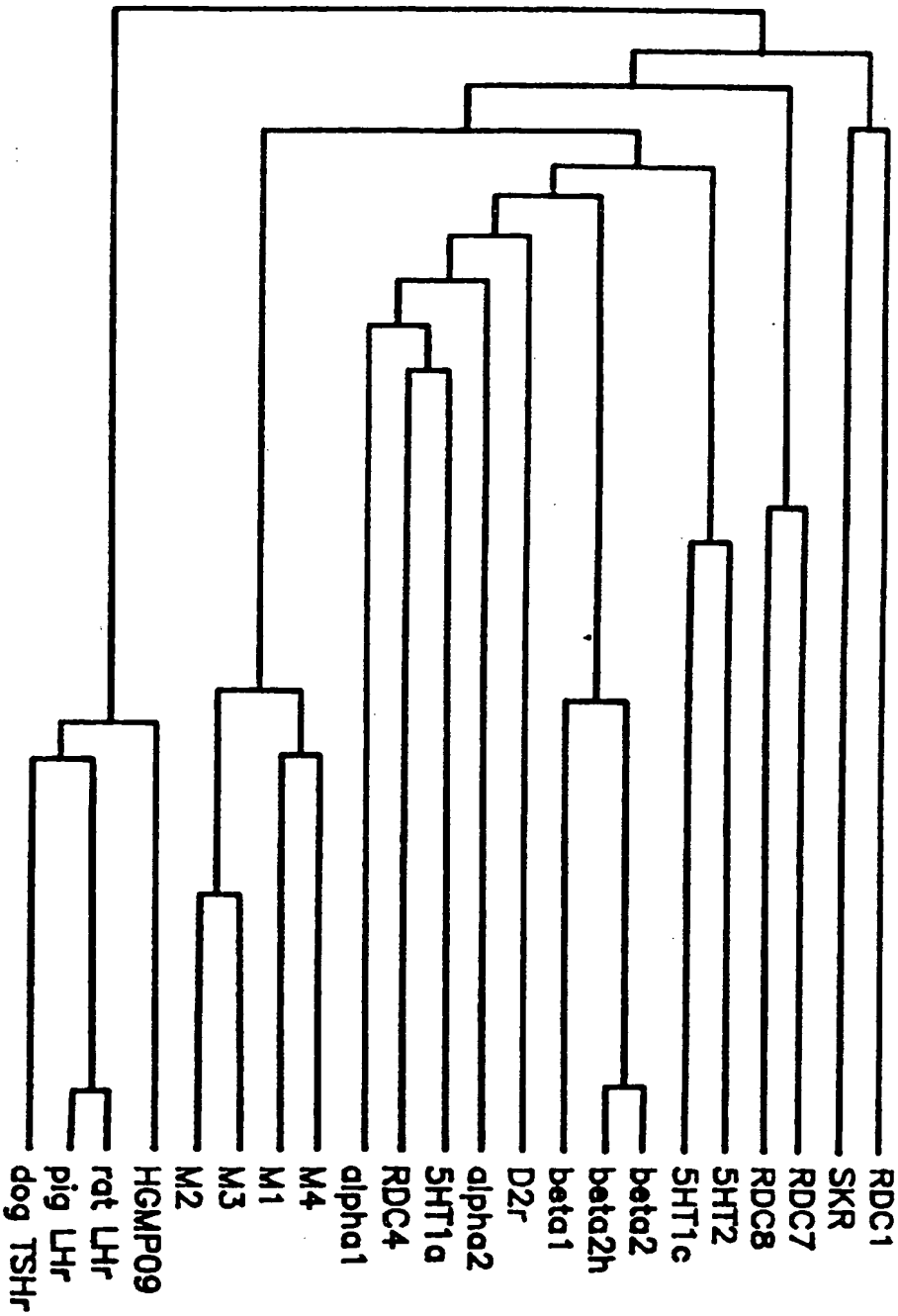

dog TSHr
pig LHr
rat LHr
HGMP09
M2
M3
M1
M4
alpha1
RDC4
5HT1a
alpha2
D2r
beta1
beta2h
beta2
5HT1c
5HT2
RDC8
RDC7
SKR
RDC1

Fig 3a

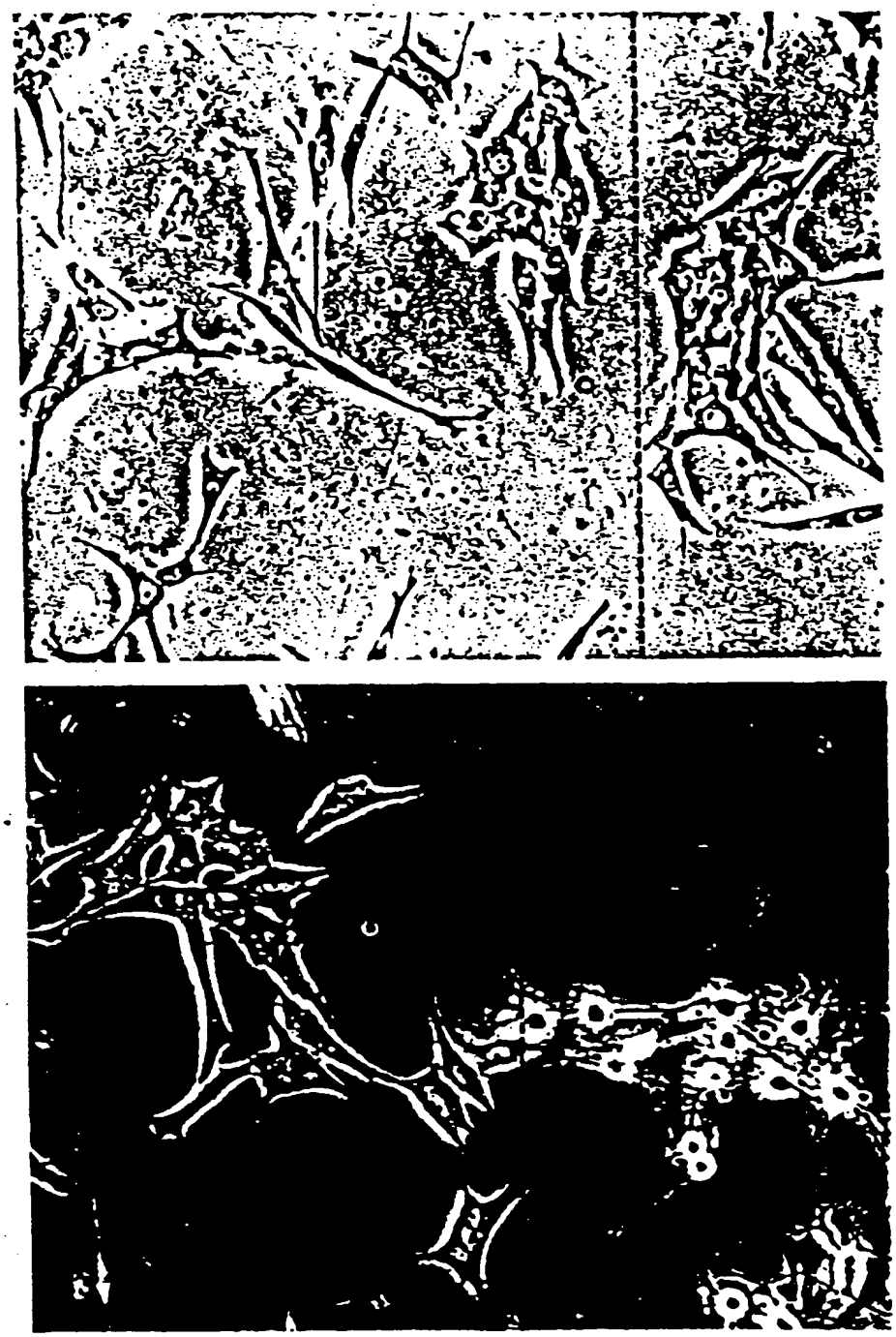


Fig 3b

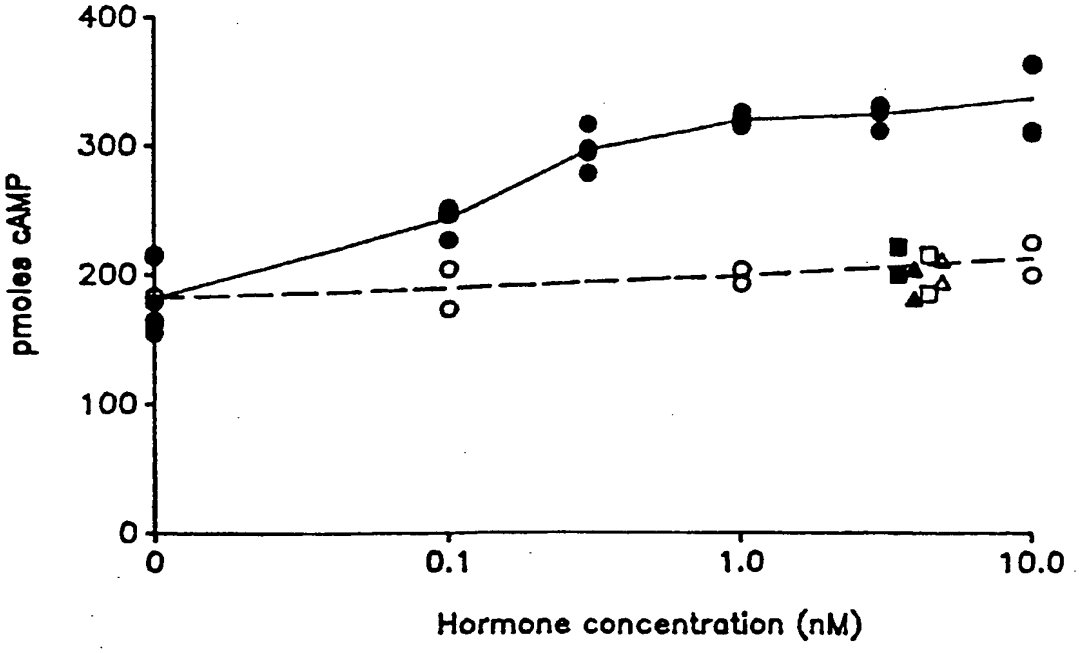
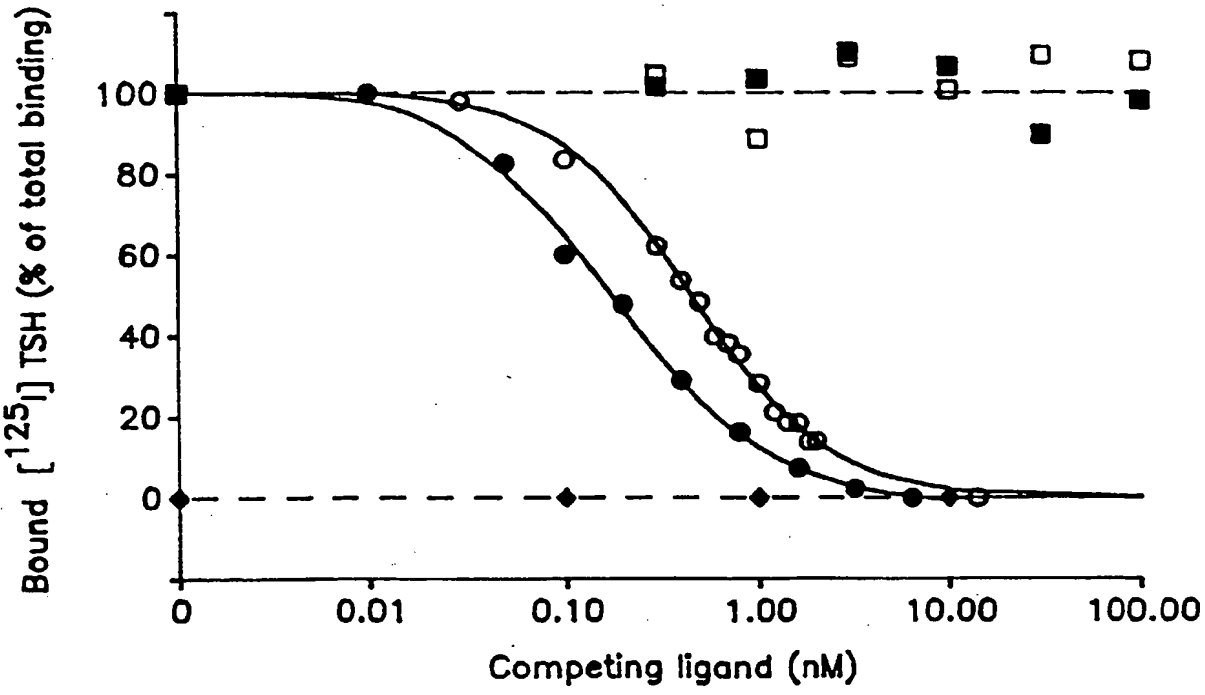

400
300
200
100
0
pmoles cAMP
0
0.1
1.0
10.0
Hormone concentration (nM)

Fig 4


Bound [125I] TSH (% of total binding)
100
80
60
40
20
0
0
0.01
0.10
1.00
10.00
100.00
Competing ligand (nM)

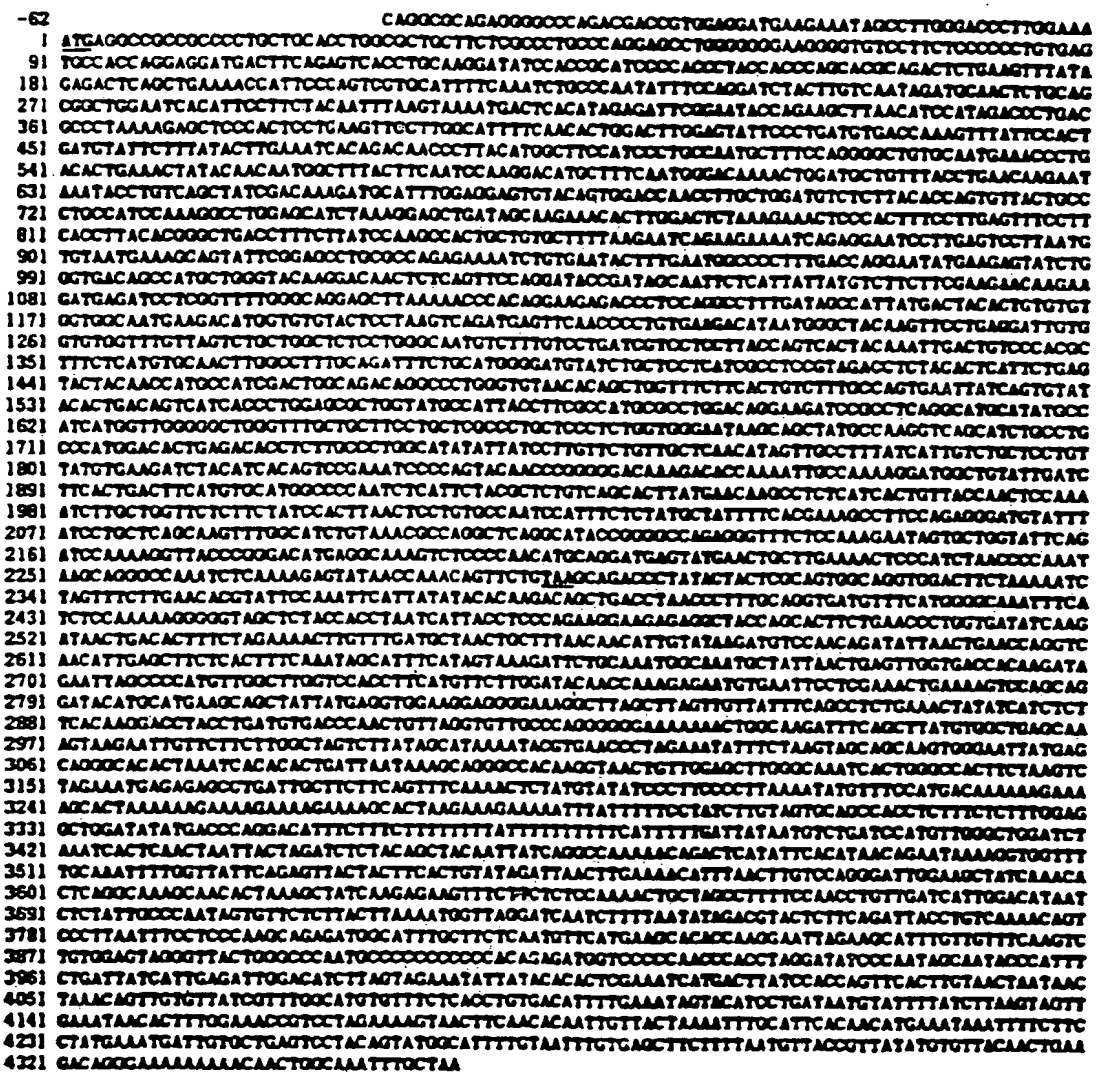


Fig 5

Fig 6.

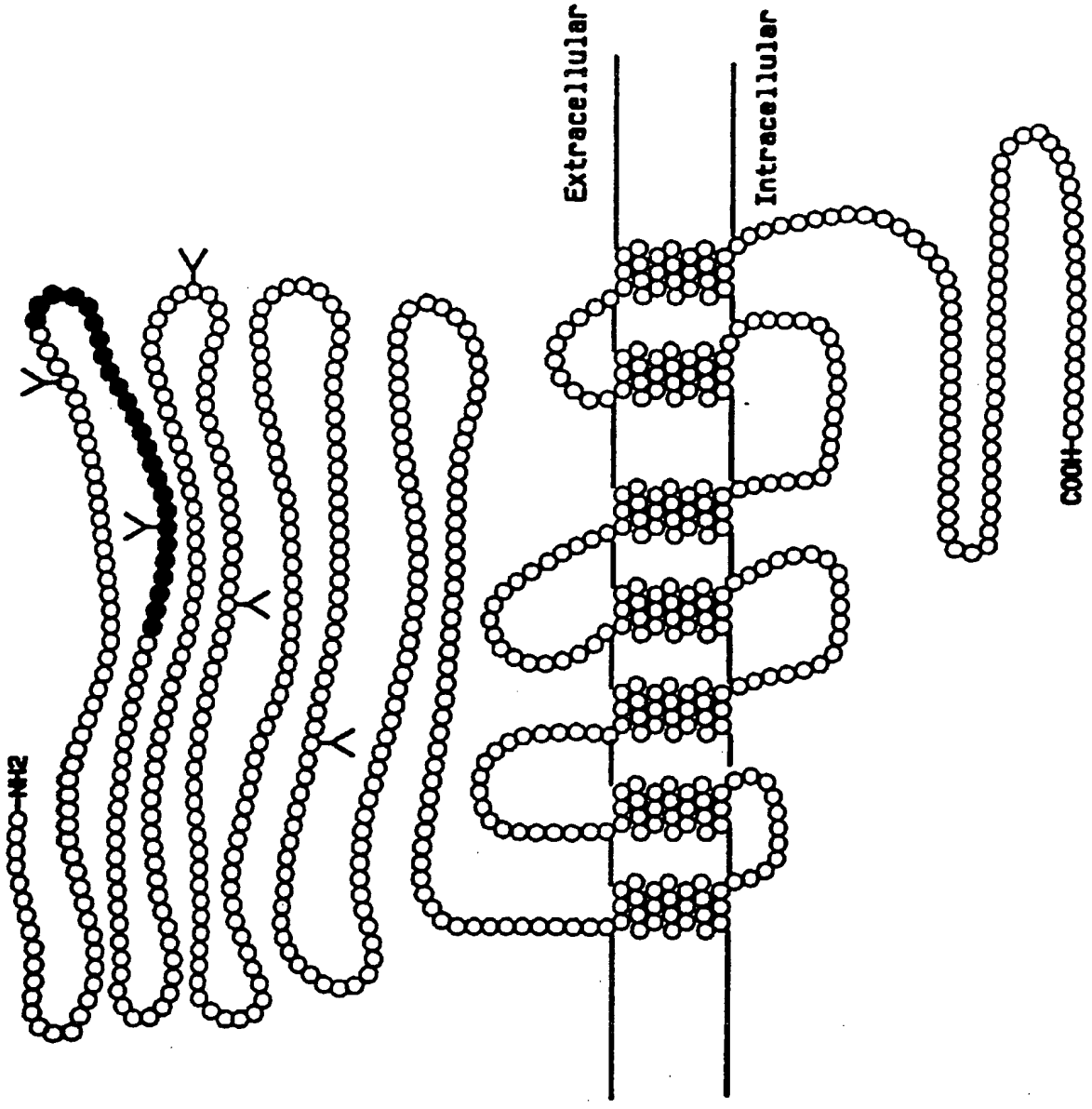


Fig 7

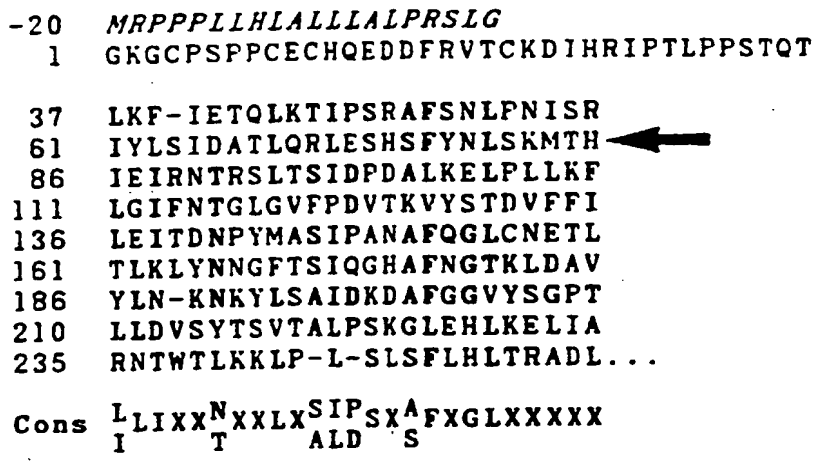

-20 MRPPPLLHLALLLALPRSLG
1 GKGCPSPPCECHQEDDFRVTCKDIHRIPTLPPSTQT
37 LKF-IETQLKTIPSRAFSNLPNISR
61 IYLSIDATLQRLESHSFYNLSKMTH
86 IEIRNTRSLTSIDPDALKELPLLKF
111 LGIFNTGLGVFPDVTKVYSTDVFFI
136 LEITDNPYMASIPANAFQGLCNETL
161 TLKLYNNGFTSIQGHAFNGTKLDAV
186 YLN-KNKYLSAIDKDAFGGVYSGPT
210 LLDVSYTSVTALPSKGLEHLKELIA
235 RNTWTLKKLP-L-SLSFLHLTRADL...
Cons LIXXNXXLXSIPSXAFXGLXXXXX
I T ALD S

Fig 8

Comparison of human and dog TSH receptor sequences

```
                -20                 1                  20                  40
Human TSHR :  MRPADLLQLVLLLDLPRDLGGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLI
Dog TSHR   :     PP  H A   A   S  K  P          D          H   T          F
                           60                  80                  100
Human TSHR :  ETHLRTIPSHAFSNLPNISRIYVSIDLTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPD
Dog TSHR   :    Q K    R             L   A   R          M          S  S
                          120                 140                 160
Human TSHR :  ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETL
Dog TSHR   :                GV   V       V              A   A
                          180                 200                 220
Human TSHR :  TLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTA
Dog TSHR   :             I  H                SA             T     Y
                          240                 260                 280
Human TSHR :  LPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGILESLM
Dog TSHR   :
                          300                 320                 340
Human TSHR :  CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQE
Dog TSHR   :      IR          T  G FD      Y    HA   DN Q    DS S
                          360                 380                 400
Human TSHR :  DEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCEDIMGYKFLRIV
Dog TSHR   :    L                          V  GN
              I           420                 440    II           460
Human TSHR :  VWFVSLLALLGNVFVLLILLTSHYKLNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSE
Dog TSHR   :                IV          T
                          480        III      500                 520
Human TSHR :  YYNHAIDWQTGPGCNTAGFFTVFASELSVYTLTVITLERWYAITFAMRLDRKIRLRHACA
Dog TSHR   :                                                          Y
           IV             540                 560          V        580
Human TSHR :  IMVGGWVCCFLLALLPLVGISSYAKVSICLPMDTETPLALAYIVFVLTLNIVAFVIVCCC
Dog TSHR   :                                                IL  L      I   S
                          600          VI     620                      640
Human TSHR :  YVKIYITVRNPQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLITVSNSK
Dog TSHR   :                                      M              LM       T
           VII            660                 680                 700
Human TSHR :  ILLVLFYPLNSCANPFLYAIFTKAFQRDVFILLSKFGICKRQAQAYRGQRVPPKNSTDIQ
Dog TSHR   :                                                          S    AG
                          720                 740
Human TSHR :  VQKVTHDMRQGLHNMEDVYELIENSHLTPKKQGQISEEYMQTVL
Dog TSHR   :  I    R    S P  Q E   L       N      K  N
```

Fig 9

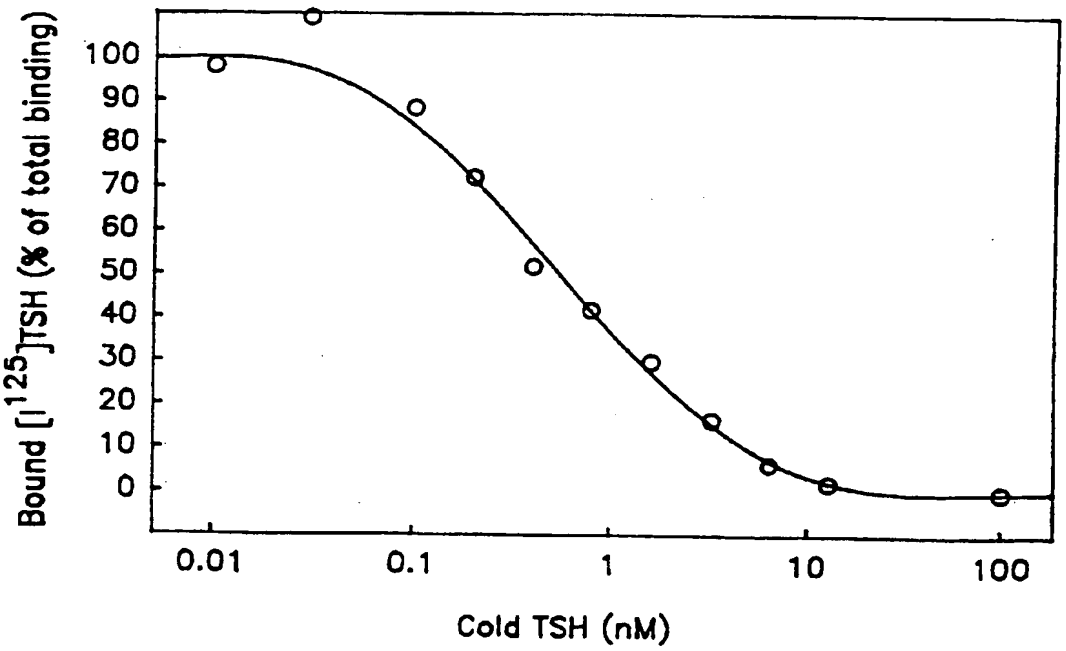
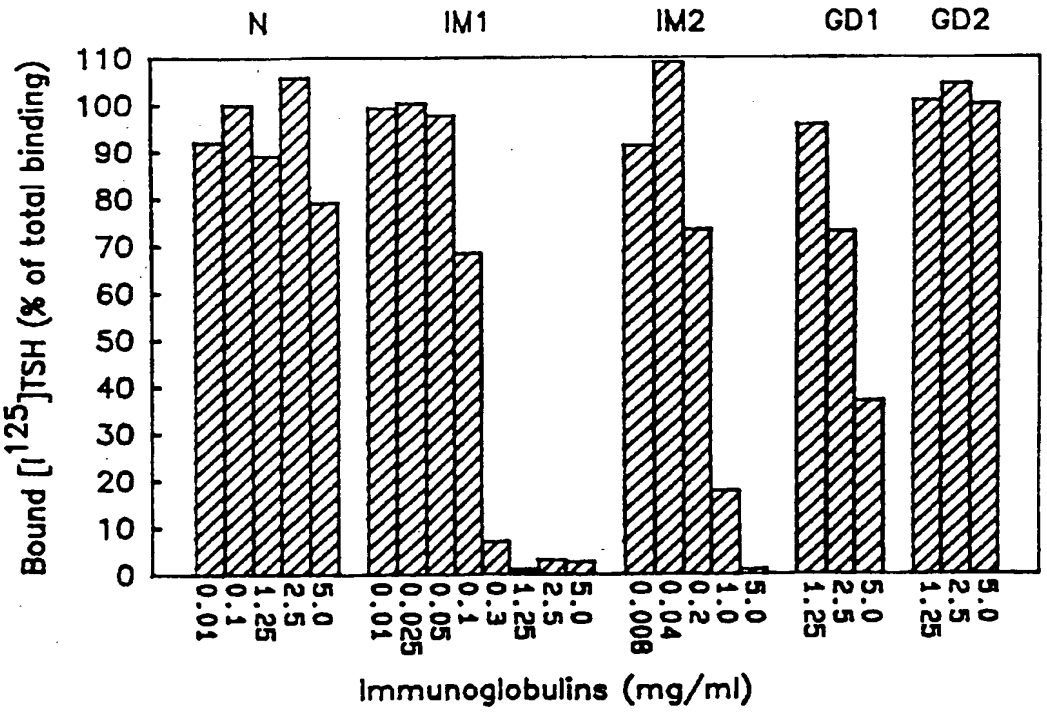

Bound [I125]TSH (% of total binding)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
Cold TSH (nM)

Fig 10


N
IM1
IM2
GD1
GD2
Bound [I125]TSH (% of total binding)
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1.25
2.5
5.0
0.01
0.025
0.05
0.1
0.3
1.25
2.5
5.0
0.008
0.04
0.2
1.0
5.0
1.25
2.5
5.0
1.25
2.5
5.0
Immunoglobulins (mg/ml)

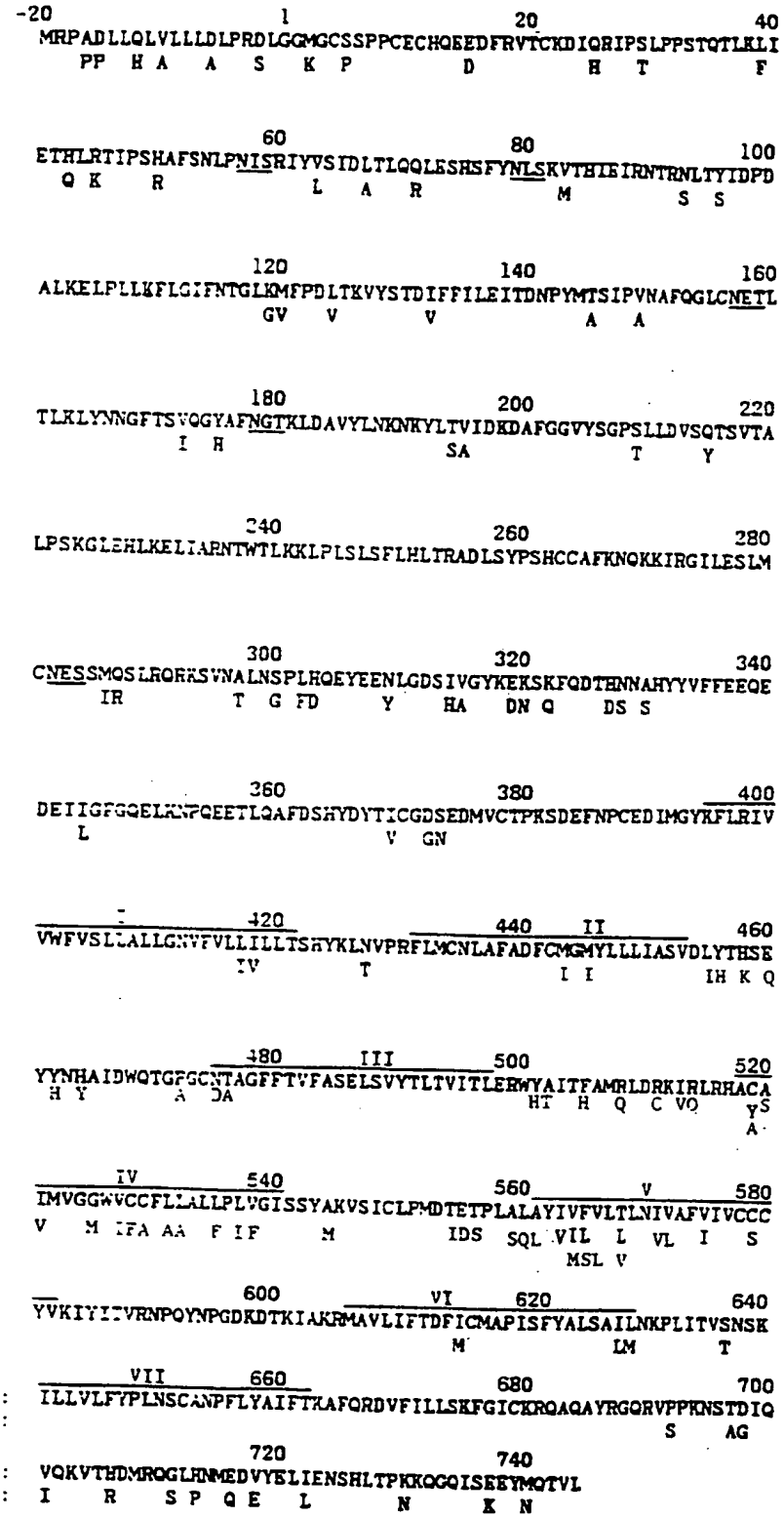


Fig 11

Fig 12

```
          10         20         30         40         50         60         70
5' AGGCAGCAGTTTCCTCCTGGGACCTGATGGCTCCCAGATCACTATCTTGGGCCCAGACTTTCTGGAGCTG
          80         90        100        110        120        130        140
AATCTCCAGTTGCCTCGGAGCCTCCTCAGACTCAGTGTGGCCAGAATGGTGGTCCTGGCTTCCCCTCGGG
         150        160        170        180        190        200        210
CCTGCCCTTCTGCCTCCTTCTGCACCCTGAGATGGTCATCAGCTTTTCTCCCACTGCTGCCCTGTATGCA
         220        230        240        250        260        270        280
GGGAAGGCCTGCCTGTGGCTGTATCTGTAGTACTTCTTGAATGTGTTTCCTTCTCCCCCAGGCCAGAGCT
         290        300        310        320        330        340        350
GAGAATGAGGCGATTTCGGAGGATGGAGAAATAGCCCCGAGTCCCGTGGAAAATGAGGCCGGCGGACTTG
         360        370        380        390        400        410        420
CTGCAGCTGGTGCTGCTGCTCGACCTGCCCAGGGACCTGGGCGGAATGGGGTGTTCGTCTCCACCCTGCG
         430        440        450        460        470        480        490
AGTGCCATCAGGAGGAGGACTTCAGAGTCACCTGCAAGGATATTCAACGCATCCCCAGCTTACCGCCCAG
         500        510        520        530        540        550        560
TACGCAGACTCTGAAGCTTATTGAGACTCACCTGAGAACTATTCCAAGTCATGCATTTTCTAATCTGCCC
         570        580        590        600        610        620        630
AATATTTCCAGAATCTACGTATCTATAGATCTGACTCTGCAGCAGCTGGAATCACACTCCTTCTACAATT
         640        650        660        670        680        690        700
TGAGTAAAGTGACTCACATAGAAATTCGGAATACCAGGAACTTAACTTACATAGACCCTGATGCCCTCAA
         710        720        730        740        750        760        770
AGAGCTCCCCCTCCTAAAGTTCCTTGGCATTTTCAACACTGGACTTAAAATGTTCCCTGACCTGACCAAA
         780        790        800        810        820        830        840
GTTTATTCCACTGATATATTCTTTATACTTGAAATTACAGACAACCCTTACATGACGTCAATCCCTGTGA
         850        860        870        880        890        900        910
ATGCTTTTCAGGGACTATGCAATGAAACCTTGACACTGAAGCTGTACAACAATGGCTTTACTTCAGTCCA
         920        930        940        950        960        970        980
AGGATATGCTTTCAATGGGACAAAGCTGGATGCTGTTTACCTAAACAAGAATAAATACCTGACAGTTATT
         990       1000       1010       1020       1030       1040       1050
GACAAAGATGCATTTGGAGGAGTATACAGTGGACCAAGCTTGCTGGACGTGTCTCAAACCAGTGTCACTG
        1060       1070       1080       1090       1100       1110       1120
CCCTTCCATCCAAAGGCCTGGAGCACCTGAAGGAACTGATAGCAAGAAACACCTGGACTCTTAAGAAACT
        1130       1140       1150       1160       1170       1180       1190
TCCACTTTCCTTGAGTTTCCTTCACCTCACACGGGCTGACCTTTCTTACCCAAGCCACTGCTGTGCTTTT
        1200       1210       1220       1230       1240       1250       1260
AAGAATCAGAAGAAAATCAGAGGAATCCTTGAGTCCTTGATGTGTAATGAGAGCAGTATGCAGAGCTTGC
        1270       1280       1290       1300       1310       1320       1330
GCCAGAGAAAATCTGTGAATGCCTTGAATAGCCCCCTCCACCAGGAATATGAAGAGAATCTGGGTGACAG
        1340       1350       1360       1370       1380       1390       1400
CATTGTTGGGTACAAGGAAAAGTCCAAGTTCCAGGATACTCATAACAACGCTCATTATTACGTCTTCTTT
        1410       1420       1430       1440       1450       1460       1470
GAAGAACAAGAGGATGAGATCATTGGTTTTGGCCAGGAGCTCAAAAACCCCCAGGAAGAGACTCTACAAG
        1480       1490       1500       1510       1520       1530       1540
CTTTTGACAGCCATTATGACTACACCATATGTGGGGACAGTGAAGACATGGTGTGTACCCCCAAGTCCGA
        1550       1560       1570       1580       1590       1600       1610
TGAGTTCAACCCGTGTGAAGACATAATGGGCTACAAGTTCCTGAGAATTGTGGTGTGGTTCGTTAGTCTG
        1620       1630       1640       1650       1660       1670       1680
CTGGCTCTCCTGGGCAATGTCTTTGTCCTGCTTATTCTCCTCACCAGCCACTACAAACTGAACGTCCCCC
        1690       1700       1710       1720       1730       1740       1750
GCTTTCTCATGTGCAACCTGGCCTTTGCGGATTTCTGCATGGGGATGTACCTGCTCCTCATCGCCTCTGT
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Fig 12 suite

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      1760       1770       1780       1790       1800       1810       1820
AGACCTCTACACTCACTCTGAGTACTACAACCATGCCATCGACTGGCAGACAGGCCCTGGGTGCAACACG
      1830       1840       1850       1860       1870       1880       1890
GCTGGTTTCTTCACTGTCTTTGCAAGCGAGTTATCGGTGTATACGCTGACGGTCATCACCCTGGAGCGCT
      1900       1910       1920       1930       1940       1950       1960
GGTATGCCATCACCTTCGCCATGCGCCTGGACCGGAAGATCCGCCTCAGGCACGCATGTGCCATCATGGT
      1970       1980       1990       2000       2010       2020       2030
TGGGGGCTGGGTTTGCTGCTTCCTTCTCGCCCTGCTTCCTTTGGTGGGAATAAGTAGCTATGCCAAAGTC
      2040       2050       2060       2070       2080       2090       2100
AGTATCTGCCTGCCCATGGACACCGAGACCCCTCTTGCTCTGGCATATATTGTTTTTGTTCTGACGCTCA
      2110       2120       2130       2140       2150       2160       2170
ACATAGTTGCCTTCGTCATCGTCTGCTGCTGTTATGTGAAGATCTACATCACAGTCCGAAATCCGCAGTA
      2180       2190       2200       2210       2220       2230       2240
CAACCCAGGGGACAAAGATACCAAAATTGCCAAGAGGATGGCTGTGTTGATCTTCACCGACTTCATATGC
      2250       2260       2270       2280       2290       2300       2310
ATGGCCCCAATCTCATTCTATGCTCTGTCAGCAATTCTGAACAAGCCTCTCATCACTGTTAGCAACTCCA
      2320       2330       2340       2350       2360       2370       2380
AAATCTTGCTGGTACTCTTCTATCCACTTAACTCCTGTGCCAATCCATTCCTCTATGCTATTTTCACCAA
      2390       2400       2410       2420       2430       2440       2450
GGCCTTCCAGAGGGATGTGTTCATCCTACTCAGCAAGTTTGGCATCTGTAAACGCCAGGCTCAGGCATAC
      2460       2470       2480       2490       2500       2510       2520
CGGGGGCAGAGGGTTCCTCCAAAGAACAGCACTGATATTCAGGTTCAAAAGGTTACCCACGACATGAGGC
      2530       2540       2550       2560       2570       2580       2590
AGGGTCTCCACAACATGGAAGATGTCTATGAACTGATTGAAAACTCCCATCTAACCCCAAAGAAGCAAGG
      2600       2610       2620       2630       2640       2650       2660
CCAAATCTCAGAAGAGTATATGCAAACGGTTTTGTAAGTTAACACTACACTACTCACAATGCTAGGGGAA
      2670       2680       2690       2700       2710       2720       2730
CTTACAAAATAATAGTTTCTTGAATATGCATTCCAATCCCATGACACCCCCAACACATAGCTGCCCTCAC
      2740       2750       2760       2770       2780       2790       2800
TCTTGTGCAGGCGATGTTTCAATGTTTCATGGGGCAAGAGTTTATCTCTGGAGAGTGATTAGTATTAACC
      2810       2820       2830       2840       2850       2860       2870
TAATCATTGCCCCCAAGAAGGAAGTTAGGCTACCAGCATATTTGAATGCCAGGTGAAATCAAAATAATCT
      2880       2890       2900       2910       2920       2930       2940
ACACTATCTAGAAGACTTTCTTGATGCCAAGTCCAGAGATGTCATTGTGTAGGATGTTCAGTAAATATTA
      2950       2960       2970       2980       2990       3000       3010
ACTGAGCTATGTCAATATAGAGCTTCTCAGTTTTGTATAACATTTCATACTAAAGATTCAGCAAATGGAA
      3020       3030       3040       3050       3060       3070       3080
AATGCTATTAATTTGGTTGGTGACCACAAGATAAAATCAGTCCCACGTTGGCTCAGTTCAACTAGATGTT
      3090       3100       3110       3120       3130       3140       3150
CCCTGATACAAAGAGAACTTGATTTCCTTAAAACTGAAAAGCCAAACACAGCTAGCTGTCATACAAGAAA
      3160       3170       3180       3190       3200       3210       3220
CAGCTATTATGAGACATGAAGGAGGGTAAGAATTAGCTTTAAGTTTTGTTTTGCTTTGTTTTGTTTTTTA
      3230       3240       3250       3260       3270       3280       3290
ACTCAACCTATTAATCATCTCTTCACAAGAATCCACCTGATGTGACCAAGCTATTATGTGTTGCCTGGAA
      3300       3310       3320       3330       3340       3350       3360
AAACTGGCAAGATTTCAGCTTATGTGGCCTAGCAAACTAAGAATTGCTCTTCTTGGCCAGCCTCATAGCA
      3370       3380       3390       3400       3410       3420       3430
TAAAAGATGTGAACTCTAGGAAGTCTTTCTGAGTAGCAATAAGTGGGAATTATGGGCAGAGCACACTCAA
      3440       3450       3460       3470       3480       3490       3500
TCCCCTGTTGATTAATAAAACAGGCTGGACACTAATTAACTATGGGACTTAAATCTGTAGAAATGAAGGA
      3510       3520       3530       3540       3550       3560       3570
GTCCAATAGCTTCTTCCAATTTTAAAACTCTAGTACATCCCTTTCCCTCAAATATATATTTCTAAGATAA
      3580       3590       3600       3610       3620       3630       3640
AGAGAAAGAAGAGCACTAAGTAAGTAGAATCTGTTTTTCCTATTTTGTAGGGCTGCTGACTCCTAGTCCT
      3650       3660       3670       3680       3690       3700       3710
TGAAGCCTAGACACATGACCCAGGAAATTTTCCTTTGTTTCACTTTTGATTATGATGTCTGAGCCAAAAA 3'
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